Background/Summary

RELATED APPLICATIONS [0001] Benefit of priority is claimed to(1) This application is a divisional of U.S. application Ser. No. 13/694,731, entitled "PH20 POLYPEPTIDE VARIANTS, FORMULATIONS AND USES THEREOF," and filed Dec. 28, 2012, to Ge Wei, H. Michael Shepard, Qiping Zhao and Robert James Connor, now U.S. Pat. No. 9,447,401, which claims the benefit of U.S. Provisional Application NoNos. 61/631,313 and 61/796,208, filed on Dec. 30, 2011, and to U.S. Provisional Application No. 61/796,208 filed Nov. 1, 2012, respectively, and each entitled ""PH20 Polypeptide Variants, Formulations and Uses Thereof." [0002]POLYPEPTIDE VARIANTS, FORMULATIONS AND USES THEREOF." (2) This application also is related to International PCT Application Serial No. (Attorney Docket No. 33320.03087.WO01/3087PC), filed the same day herewith, entitled "PH20 Polypeptide Variants, Formulations and Uses Thereof," whichPCT/US2012/072182, filed Dec. 28, 2012, entitled "PH20 POLYPEPTIDE VARIANTS, FORMULATIONS AND USES THEREOF," which also claims priority to U.S. Provisional Application NoNos. 61/631,313 and U.S. Provisional Application No. 61/796,208. [0003](3) The subject matter of each of the above-noted related applications is incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING PROVIDED ON COMPACT DISCSELECTRONICALLY

[0004](1) An electronic version on compact disc (CD-R) of the Sequence Listing is filed herewith in duplicate (labeled Copy #1 and Copy #2), the contents of which are incorporated by reference in their entirety. The computer-readable file on each of the aforementioned compact discs,electronic file was created on DecJul. 28, 20122016, is identical, 3.483.59 megabytes in size, and titled 3087seq.0013087Bseq001.txt. A substitute Sequence Listing, is filed electronically herewith, the contents of which are incorporated by reference in its entirety, is provided on identical compact discs (labeled Copy 1 Replacement Mar. 20, 2013, Copy 2 Replacement Mar. 20, 2013). The computer-readable file on each of the aforementioned compact discs,their entirety. The electronic file was created on MarAug. 2025, 20132016, is identical, 3.503.59 megabytes in size, and titled 3087seq.002.txt. A substitute Sequence Listing, incorporated by reference in its entirety, is provided on identical compact discs (labeled Copy 1 Replacement Apr. 18, 2013, Copy 2 Replacement Apr. 18, 2013). The computer-readable file on each of the aforementioned compact discs, created on Apr. 18, 2013, is identical, 3.50 megabytes in size, and titled 3087seq.003.txt.3087Bseq002.txt.

FIELD OF THE INVENTION

[0005](2) Modified PH20 hyaluronidase polypeptides, including modified polypeptides that exhibit increased stability and/or increased activity, are provided. Also provided are compositions and formulations and uses thereof.

BACKGROUND

[0006](3) Hyaluronan (hyaluronic acid; HA) is a polypeptide that is found in the extracellular matrix of many cells, especially in soft connective tissues. HA also is found predominantly in

skin, cartilage, and in synovial fluid in mammals. Hyaluronan also is the main constituent of the vitreous of the eye. HA has a role in various physiological processes, such as in water and plasma protein homeostasis (Laurent T C et al. (1992) FASEB J 6: 2397-2404). Certain diseases are associated with expression and/or production of hyaluronan. Hyaluronan-degrading enzymes, such as hyaluronidases, are enzymes that degrade hyaluronan. By catalyzing HA degradation, hyaluronan-degrading enzymes (e.g., hyaluronidases) can be used to treat diseases or disorders associated with accumulation of HA or other glycosaminoglycans. Also, since HA is a major component of the interstitial barrier, hyaluronan-degrading enzymes (e.g., hyaluronidase) increase tissue permeability and therefore can be used to increase the dispersion and delivery of therapeutic agents. Various hyaluronidases have been used therapeutically (e.g., Hydase~~.TM.~~® (bovine testicular hyaluronidase), Vitrase~~.TM.~~® (ovine hyaluronidase), and Wydase~~.TM.~~® (bovine hyaluronidase)), typically as dispersing and spreading agents in combination with other therapeutic agents. Many of these are ovine or bovine forms, which can be immunogenic for treatment of humans. Improved hyaluronan-degrading enzymes, such as hyaluronidases, and compositions thereof that can be used for treatment are needed.

SUMMARY

~~[0007]~~(4) Provided are modified PH20 polypeptides that have an altered property or properties compared to the PH20 polypeptide that do not have the modification(s). The modifications include amino acid replacement, deletion and/or insertions. Detailed structure/function of virtually each amino acid in a PH20 polypeptide is provided herein, as well as the identification of residues and loci that contribute to alteration of a property, such as stability in particular conditions, is provided. Hence, provided are modified PH20 polypeptides that contain one or more amino acid replacements that result in a PH20 polypeptide that retains activity and/or exhibits increased or altered stability under a variety of conditions. Activity retained can be, for example, hyaluronidase activity that is as least about 40% or more of the PH20 polypeptide that does not include the replacement. Exemplary modifications are amino acid replacements. For purposes herein, amino acid replacements are denoted by the single amino acid letter followed by the corresponding amino acid position in SEQ ID NO:3 in which the replacement occurs. Single amino acid abbreviations for amino acid residues are well known to a skilled artisan (see e.g. Table 1), and are used herein throughout the description and examples. For example, replacement with P at a position corresponding to position 204 in a PH20 polypeptide with reference to amino acid residue positions set forth in SEQ ID NO:3 means that the replacement encompasses F204P in a PH20 polypeptide set forth in SEQ ID NO:3, or the same replacement at the corresponding position in another PH20 polypeptide.

~~[0008]~~(5) Provided are modified PH20 polypeptides that contain at least one amino acid replacement in a PH20 polypeptide, whereby the modified PH20 polypeptide exhibits increased stability compared to the PH20 polypeptide not containing the amino acid replacement. Increased stability can be manifested as increased resistance to one or more protein conditions that are denaturing to proteins. The stability of modified and unmodified PH20 is compared under the same conditions. Exemplary protein denaturation (or denaturing, used interchangeably herein) conditions include, but are not limited to, elevated temperature greater than 30~~.degree.~~° C. or about 30~~.degree.~~° C., agitation, low salt, including essentially or substantially or no salt, and presence of excipients that tend to denature proteins. Exemplary of such excipients are antiadherent(s), ~~binders~~binder(s), coating(s), ~~fillers~~filler(s) and diluent(s), ~~flavors~~flavor(s),

color(s), lubricant(s), glidant(s), preservative(s), detergent(s), sorbent(s) and combinations thereof.

~~[0009]~~(6) The modified PH20 polypeptide can be one in which the unmodified form thereof has at least about 68% sequence identity to SEQ ID NO: 3 and further contains modifications that alter stability and/or can be a PH20 polypeptide that includes as many as about up to 100, 110, 120, 130, 150 amino acid differences from PH20 but retains enzymatic activity, particularly, at least about 40% of the activity of the unmodified PH20 polypeptide and exhibits increased stability, such as stability under denaturing conditions. Thus, included are modified PH20 polypeptides that have at least 68% or about 68% amino acid sequence identity to the sequence of amino acids set forth in SEQ ID NO:3. Included are modified PH20 polypeptides that have at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%~~)~~, 95%, 96%, 97%, 98% or 99% amino acid sequence identity to the sequence of amino acids set forth in SEQ ID NO:3. Exemplary of such modified PH20 polypeptides are polypeptides that contain amino acid replacement(s) in a PH20 polypeptide that contains the sequence of amino acid residues as set forth in any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, 870 or a sequence of amino acids that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, or 870.

~~[0010]~~(7) For example, provided herein is a modified PH20 polypeptide that exhibits increased stability containing an amino acid replacement in a PH20 polypeptide that confers the increased stability, wherein increased stability is manifested as increased resistance to denaturation in the presence of one or more protein denaturation conditions, stability is increased compared to the PH20 polypeptide not containing the amino acid replacement, and the unmodified PH20 polypeptide consists of the sequence of amino acids set forth in SEQ ID ~~NOS~~NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide or has at least 85% sequence identity thereto. As above, the modified PH20 polypeptide that exhibits increased stability exhibits increased stability to a denaturation condition that is temperature greater than or about 30~~.degree.~~° C.; agitation; low or no a salt; or presence of an excipient or a denaturing agent, such as an antiadherent(s), ~~binders~~binder(s), coating(s), ~~fillers~~filler(s) and diluent(s), flavor(s), color(s), lubricant(s), glidant(s), preservative(s), detergent(s), sorbent(s) or ~~sweeteners~~sweetener(s) and a combination thereof, and in particular a preservative. In some examples of such modified PH20 polypeptides that exhibit increased stability, the ~~denatutation~~denaturation condition is temperature greater than 30~~.degree.~~° C., and the modified PH20 polypeptide exhibits greater hyaluronidase activity at the temperature compared to the unmodified PH20 polypeptide not containing the amino acid replacement(s) where the activities are compared under the same conditions. In other examples, the protein denaturation condition is the presence of low concentrations of salt of less than 100 mM, and the modified PH20 polypeptide exhibits increased hyaluronidase activity in the presence of low concentrations of salt compared to the unmodified PH20 polypeptide not containing the amino acid replacement(s) where the activities are compared under the same conditions.

~~[0011]~~(8) In any of the above examples of a modified PH20 polypeptide that exhibits increased stability, stability can be assessed based on a variety of parameters including hyaluronidase activity, solubility, aggregation and/or crystallization. Stability can be assessed in the presence of a denaturing condition. When stability of two or more polypeptides is compared, stability is

assessed under the same conditions. In some instances, among the PH20 polypeptides provided herein, the modified PH20 polypeptide exhibits at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement(s).

~~[0012]~~(9) In any of the above examples of a modified PH20 polypeptide that exhibits increased stability, ~~tenaturing~~denaturing conditions include the presence of excipients that denature proteins. Exemplary of such conditions is the presence of a preservative, such as a phenolic preservative. Provided are modified PH20 polypeptides that exhibit increased stability in the presence of an anti-microbial effective amount of one or more phenolic preservatives. An anti-microbial effective amount is the total amount of one or more phenolic preservative agents, which can be expressed as a percentage (%) of mass concentration (w/v) that is or is between (or at least about or at about) 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4%, inclusive. Exemplary phenolic preservatives include, but are not limited to, phenol, metacresol (m-cresol), benzyl alcohol, and a paraben, such as methylparaben propylparaben, m-cresol, phenol or m-cresol and phenol. Exemplary of the stability achieved by provided modified PH20 polypeptides are those that exhibit at least 15% or about 15% of the hyaluronidase activity for at least 4 hours in the presence of preservative(s) compared to the modified PH20 polypeptide in absence of preservative. Activity is compared under the same conditions except for the presence of preservative(s). For example, provided are modified PH20 polypeptides that exhibit at least (or at least about) 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the hyaluronidase activity in the presence of a phenolic preservative(s) compared to absence of the same preservative(s). Thus, provided, among the modified PH20 polypeptides provided herein, are PH20 polypeptides that, by virtue of amino acid replacement(s), are phenophilic compared to PH20 polypeptides without such replacement. Included are modified PH20 polypeptides where the hyaluronidase activity is exhibited after at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks or more in the presence of the preservative(s) compared to the hyaluronidase activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions except for the presence of preservative(s).

~~[0013]~~(10) In examples of a modified PH20 polypeptide that exhibits increased stability to a phenolic preservative, increased stability in a phenolic preservative can be exhibited under temperature conditions that include any temperature between, for example, 0~~.degree.~~° C. and 40~~.degree.~~° C., such as between or about between 0~~.degree.~~° C. to 40~~.degree.~~° C., 2~~.degree.~~° C. to 6~~.degree.~~° C., 24~~.degree.~~° C. to 32~~.degree.~~° C. and 35~~.degree.~~° C. to 40~~.degree.~~° C. Exemplary polypeptides exhibit increased stability at temperatures of between or about between 30~~.degree.~~° C. to 45~~.degree.~~° C., 35~~.degree.~~° C. to 45~~.degree.~~° C., 30~~.degree.~~° C. to 37~~.degree.~~° C., 35~~.degree.~~° C. to 37~~.degree.~~° C. or 37~~.degree.~~° C. to 42~~.degree.~~° C., each inclusive. The particular modified PH20 polypeptide and conditions depend upon the intended formulation, conditions to which the formulation will be exposed and/or intended application.

[0014](11) Particular and exemplary modified PH20 polypeptides that exhibit increased stability, such as increased stability to a phenolic preservative, include those that contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. These include modified PH20 polypeptides that contain one or more amino acid replacements, where at least one replacement is at an amino acid position corresponding (i.e., by alignment) to a position selected from among 10, 12, 20, 22, 26, 34, 36, 46, 50, 52, 58, 68, 70, 74, 82, 83, 84, 86, 97, 127, 131, 138, 142, 143, 144, 166, 169, 174, 193, 195, 196, 204, 205, 206, 213, 219, 234, 237, 238, 240, 249, 261, 267, 277, 279, 291, 309, 310, 314, 315, 317, 318, 347, 367, 375, 376, 399, 401, 407, 416, 419, 421, 431, 433, 439, 440, 443 or 445 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. Exemplary of such modifications are at least one amino acid replacement selected from among replacement with: glycine (G) at a position corresponding to position 10; K at a position corresponding to position 12; S at a position corresponding to position 20; T at a position corresponding to position 22; M at a position corresponding to position 26; W at a position corresponding to position 34; N at a position corresponding to position 36; L at a position corresponding to position 46; M at a position corresponding to position 50; T at a position corresponding to position 52; S at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; R at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; P at a position corresponding to position 58; H at a position corresponding to position 58; P at a position corresponding to position 68; V at a position corresponding to position 70; E at a position corresponding to position 74; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; N at a position corresponding to position 84; A at a position corresponding to position 86; K at a position corresponding to position 86; E at a position corresponding to position 97; L at a position corresponding to position 97; R at a position corresponding to position 127; R at a position corresponding to position 131; L at a position corresponding to position 138; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; T at a position corresponding to position 144; Q at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 169; G at a position corresponding to position 174; N at a position corresponding to position 174; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; E at a position corresponding to position 196; R at a position corresponding to position 196; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; I at a position corresponding to position 206; A at a position corresponding to position 213; I at a position corresponding to position 219; M at a position corresponding to position 234; T at a position corresponding to position 237; H at a position corresponding to position 238; Q at a position corresponding to position 240; V at a position corresponding to position 249; A at a position corresponding to position 261; K at a

position corresponding to position 261; T at a position corresponding to position 267; K at a position corresponding to position 277; H at a position corresponding to position 279; V at a position corresponding to position 279; V at a position corresponding to position 291; E at a position corresponding to position 309; Q at a position corresponding to position 310; Y at a position corresponding to position 314; Y at a position corresponding to position 315; N at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 375; R at a position corresponding to position 376; V at a position corresponding to position 399; E at a position corresponding to position 401; A at a position corresponding to position 407; L at a position corresponding to position 416; K at a position corresponding to position 419; H at a position corresponding to position 421; E at a position corresponding to position 431; T at a position corresponding to position 433; V at a position corresponding to position 433; C at a position corresponding to position 439; P at a position corresponding to position 440; G at a position corresponding to position 443; N at a position corresponding to position 445, with reference to amino acid residue positions set forth in SEQ ID NO:3. For example, the modified PH20 polypeptide can contain at least one amino acid replacement selected from among replacement with: T at a position corresponding to position 52, K at a position corresponding to position 58, R at a position corresponding to position 58, P at a position corresponding to position 68, V at a position corresponding to position 83, P at a position corresponding to position 204, A at a position corresponding to position 261, T at a position corresponding to position 267, K at a position corresponding to position 277 and H at a position corresponding to position 421, with reference to amino acid residue positions set forth in SEQ ID NO:3. An exemplary modified PH20 polypeptide is one that includes P (or a conservative amino acid thereto) at a position corresponding to position 204 in a PH20 polypeptide with reference to amino acid residue positions set forth in SEQ ID NO:3.

~~[0015]~~(12) Thus, provided herein are modified PH20 polypeptides that exhibit increased stability in the presence of a phenolic preservative containing an amino acid replacement in a PH20 polypeptide that confers the increased stability, wherein stability is increased compared to the unmodified polypeptide without the amino acid replacement, and the unmodified PH20 polypeptide has the sequence of amino acids set forth in SEQ ID ~~NOS~~NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide or has at least 85% sequence identity thereto. For example, the unmodified PH20 polypeptide is a soluble PH20 polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66. In particular examples, the modified PH20 polypeptide has at least 85% sequence identity to SEQ ID NO:3. In any of such examples of a modified PH20 polypeptide, the polypeptide contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75 or more amino acid replacements. In examples herein, the modified PH20 polypeptide is a human PH20. The modified PH20 polypeptide exhibits stability in the presence of phenolic preservatives if it exhibits at least 15% of the hyaluronidase activity in the presence of a preservative(s) for at least 4 hours compared to the hyaluronidase activity in the absence of the phenolic preservative(s), wherein the activity is compared under the same conditions except for the presence of the phenolic preservative(s). In any of the above examples, the modified PH20 polypeptide is stable in the presence of an of an anti-microbial effective amount of one or more phenolic preservatives, such as a total amount of

one or more phenolic preservative agents as a percentage (%) of mass concentration (w/v) that is from or from about 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4%, inclusive. The phenolic preservative can be a phenol, metacresol (m-cresol), benzyl alcohol ~~and~~or a paraben, such as m-cresol, phenol, or m-cresol and phenol. The amino acid replacement can be at amino acid residue 204, 58, 10, 12, 20, 22, 26, 34, 36, 46, 50, 52, 68, 70, 74, 82, 83, 84, 86, 97, 127, 131, 138, 142, 143, 144, 166, 169, 174, 193, 195, 196, 205, 206, 213, 219, 234, 237, 238, 240, 249, 261, 267, 277, 279, 291, 309, 310, 314, 315, 317, 318, 347, 367, 375, 376, 399, 401, 407, 416, 419, 421, 431, 433, 439, 440, 443 or 445 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. For example, the amino acid replacement is G at a position corresponding to position 10; K at a position corresponding to position 12; S at a position corresponding to position 20; T at a position corresponding to position 22; M at a position corresponding to position 26; W at a position corresponding to position 34; N at a position corresponding to position 36; L at a position corresponding to position 46; M at a position corresponding to position 50; T at a position corresponding to position 52; S at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; R at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; P at a position corresponding to position 58; H at a position corresponding to position 58; P at a position corresponding to position 68; V at a position corresponding to position 70; E at a position corresponding to position 74; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; N at a position corresponding to position 84; A at a position corresponding to position 86; K at a position corresponding to position 86; E at a position corresponding to position 97; L at a position corresponding to position 97; R at a position corresponding to position 127; R at a position corresponding to position 131; L at a position corresponding to position 138; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; T at a position corresponding to position 144; Q at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 169; G at a position corresponding to position 174; N at a position corresponding to position 174; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; E at a position corresponding to position 196; R at a position corresponding to position 196; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; I at a position corresponding to position 206; A at a position corresponding to position 213; I at a position corresponding to position 219; M at a position corresponding to position 234; T at a position corresponding to position 237; H at a position corresponding to position 238; Q at a position corresponding to position 240; V at a position corresponding to position 249; A at a position corresponding to position 261; K at a position corresponding to position 261; T at a position corresponding to position 267; K at a position corresponding to position 277; H at a position corresponding to position 279; V at a position corresponding to position 279; V at a

position corresponding to position 291; E at a position corresponding to position 309; Q at a position corresponding to position 310; Y at a position corresponding to position 314; Y at a position corresponding to position 315; N at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 375; R at a position corresponding to position 376; V at a position corresponding to position 399; E at a position corresponding to position 401; A at a position corresponding to position 407; L at a position corresponding to position 416; K at a position corresponding to position 419; H at a position corresponding to position 421; E at a position corresponding to position 431; T at a position corresponding to position 433; V at a position corresponding to position 433; C at a position corresponding to position 439; P at a position corresponding to position 440; G at a position corresponding to position 443; or N at a position corresponding to position 445, with reference to amino acid residue positions set forth in SEQ ID NO:3. In particular, the amino acid replacement is T at a position corresponding to position 52, K at a position corresponding to position 58, R at a position corresponding to position 58, P at a position corresponding to position 68, V at a position corresponding to position 83, P at a position corresponding to position 204, A at a position corresponding to position 261, T at a position corresponding to position 267, K at a position corresponding to position 277 or H at a position corresponding to position 421, with reference to amino acid residue positions set forth in SEQ ID NO:3, such as replacement with P at a position corresponding to position 204 or R at a position corresponding to position 58. The modified PH20 polypeptide that exhibits increased stability to phenolic preservatives can be substantially purified or isolated. The modified PH20 polypeptide that exhibits increased stability to phenolic preservatives can be modified by glycosylation, sialation, albumination, farnysylation, carboxylation, hydroxylation and phosphorylation, and generally is glycosylated, whereby the polypeptide contains at least an N-acetylglucosamine moiety linked to each of at least three asparagine (N) residues, such as at amino acid residues corresponding to amino acid residues 200, 333 and 358 of SEQ ID NO:3. The modified PH20 polypeptide that exhibits increased stability to phenolic ~~preserviatives~~preservatives can be conjugated to a polymer, such as PEG or dextran and/or can be conjugated to a moiety that is a multimerization domain, a toxin, a detectable label or a drug.

~~[0016]~~(13) Among modified PH20 polypeptides provided herein that exhibit increased stability are those that exhibit increased hyaluronidase activity at the elevated temperature compared to the PH20 polypeptide not containing the amino acid replacement(s), such as at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500%~~,~~ or more hyaluronidase activity for at least 4 hours compared to the PH20 polypeptide not containing the amino acid replacement(s). Also among the polypeptides are those that exhibit activity, but also typically exhibit increased stability or other property at elevated temperatures, such as a modified PH20 polypeptide that exhibits at least 95%, 96%, 97%, 98%, 99%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500% of the hyaluronidase activity for at least 4 hours at a temperature of between or about between 32~~.degree.~~° C. to 37~~.degree.~~° C. compared to the hyaluronidase activity of the modified PH20 polypeptide at a temperature of between or about between 2~~.degree.~~° C. to 8~~.degree.~~° C., where activity is compared under the same conditions except for the differences in temperature. The hyaluronidase activity can be exhibited after at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9

days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks or more at elevated temperatures of between or about between 32~~.degree.~~° C. to 37~~.degree.~~° C. compared to the hyaluronidase activity of the modified PH20 polypeptide at a temperature between or about between 2~~.degree.~~° C. to 8~~.degree.~~° C., where activity is compared for the same time period and under the same conditions except for the difference in temperature. Exemplary of such modified polypeptides are those that contain at least one amino acid replacement at an amino acid position corresponding to a position selected from among 1, 11, 12, 14, 20, 26, 29, 34, 50, 58, 70, 82, 83, 84, 86, 87, 140, 142, 143, 147, 152, 166, 167, 172, 174, 178, 193, 195, 206, 212, 213, 219, 233, 237, 240, 267, 277, 291, 292, 309, 313, 314, 317, 318, 347, 367, 368, 371, 374, 389, 392, 395, 396, 406, 419, 421, 439 and 443 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. Exemplary mutations include, for example, replacement with R at a position corresponding to position 1; S at a position corresponding to position 11; I at a position corresponding to position 12; V at a position corresponding to position 14; S at a position corresponding to position 20; M at a position corresponding to position 26; with R at a position corresponding to position 29; W at a position corresponding to position 34; M at a position corresponding to position 50; K at a position corresponding to position 58; Q at a position corresponding to position 58; Q at a position corresponding to position 58; V at a position corresponding to position 70; L at a position corresponding to position 82; Q at a position corresponding to position 83; R at a position corresponding to position 84; A at a position corresponding to position 86; S at a position corresponding to position 87; K at a position corresponding to position 140; S at a position corresponding to position 142; T at a position corresponding to position 142; K at a position corresponding to position 143; S at a position corresponding to position 147; T at a position corresponding to position 152; T at a position corresponding to position 166; D at a position corresponding to position 167; A at a position corresponding to position 172; G at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 178; Q at a position corresponding to position 193; T at a position corresponding to position 195; I at a position corresponding to position 206; S at a position corresponding to position 212; A at a position corresponding to position 213; I at a position corresponding to position 219; G at a position corresponding to position 233; T at a position corresponding to position 237; A at a position corresponding to position 240; Q at a position corresponding to position 240; T at a position corresponding to position 267; E at a position corresponding to position 277; S at a position corresponding to position 291; H at a position corresponding to position 292; V at a position corresponding to position 292; S at a position corresponding to position 309; H at a position corresponding to position 313; S at a position corresponding to position 314; I at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; R at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 368; S at a position corresponding to position 371; P at a position corresponding to position 374; A at a position corresponding to position 389; V at a position corresponding to position 392; A at a position corresponding to position 395; H at a position corresponding to position 396; N at a position corresponding to position 406; H at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 421; S at a position corresponding to position 421; A at a position corresponding to position 439; C at a position

corresponding to position 439; and G at a position corresponding to position 443, with reference to amino acid positions set forth in SEQ ID NO:3. In particular examples provided herein, any of such modified PH20 polypeptides contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. The modification, such as replacement, can be in an ~~the~~ unmodified PH20 polypeptide that has the sequence of amino acids set forth in SEQ ID ~~NOS~~NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide, such as is set forth in any of SEQ ID NOS: 3 or 32-66, or has at least 85% sequence identity thereto. For example, any of such modified PH20 polypeptides has at least 85% sequence identity to SEQ ID NO:3.

~~[0017]~~(14) Also provided are modified PH20 polypeptides that exhibit increased stability in low salt conditions, such as, for example, concentrations of NaCl of less than 100 mM, such as, but not limited to concentrations of NaCl less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less. Among the modified PH20 polypeptides are those that exhibit increased hyaluronidase activity at lower concentrations of salt compared to the PH20 polypeptide not containing the amino acid replacement(s). Such activity includes, for example, at least more than 100%, or at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500% or more hyaluronidase activity compared to the PH20 polypeptide not containing the amino acid replacement(s). Exemplary of such modified PH20 polypeptides are those that exhibit at least 60% of the hyaluronidase activity in low concentrations of salt, such as between or about between 10 mM NaCl and 100 mM NaCl, inclusive (or comparable concentrations of other salts or mixtures of salts), compared to the hyaluronidase activity of the modified PH20 polypeptide in 150 mM NaCl, where activities are compared under the same conditions except for the difference in salt concentration. In particular examples provided herein, any of such modified PH20 polypeptides contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. The modification, such as replacement, can be in an the unmodified PH20 polypeptide that has the sequence of amino acids set forth in SEQ ID ~~NOS~~NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide, such as is set forth in any of SEQ ID NOS: 3 or 32-66, or has at least 85% sequence identity thereto. For example, any of such modified PH20 polypeptides has at least 85% sequence identity to SEQ ID NO:3.

~~[0018]~~(15) Also provided are modified PH20 polypeptides that contain at least one amino acid replacement in a PH20 polypeptide, where the modified PH20 polypeptide exhibits increased hyaluronidase activity compared to the PH20 polypeptide not containing the amino acid replacement. When comparing activity among polypeptides, activity is compared under the same conditions. Among these are polypeptides, where the unmodified PH20 exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity to the sequence of amino acids set forth in SEQ ID NO:3, or the resulting modified PH20 exhibits such sequence identity to the sequence of amino acids set forth in SEQ ID NO:3. Exemplary of such modified PH20 polypeptides are any that contain an amino acid replacement(s) in the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, or 72, or a sequence of amino acids that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, or 72. The amino acid replacement(s) also

can be made in the sequence of amino acids set forth in any of SEQ ID NOS: 857, 859, 861 or 870, or a sequence of amino acids that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 857, 859, 861 or 870. In particular, provided are modified PH20 polypeptides that contain an amino acid replacement in the sequence of amino acids set forth in SEQ ID NOS: 3, 7, 32-66, 69 or 72. Among the modified PH20 polypeptides are those that that exhibit at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. Activity can be assessed at any temperature, in particular such activity is present when the hyaluronidase is exposed to a temperature that is at a temperature between or about between 2~~.degree.~~° C. to 8~~.degree.~~° C. These modified PH20 polypeptides contain at least one amino acid replacement at an amino acid position corresponding to a position selected from among 1, 12, 15, 24, 26, 27, 29, 30, 31, 32, 33, 37, 39, 46, 48, 52, 58, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 84, 86, 87, 92, 93, 94, 97, 118, 120, 127, 131, 135, 141, 142, 147, 148, 150, 151, 152, 155, 156, 163, 164, 165, 166, 169, 170, 174, 198, 206, 209, 212, 213, 215, 219, 233, 234, 236, 238, 247, 257, 259, 260, 261, 263, 269, 271, 272, 276, 277, 278, 282, 291, 293, 305, 308, 309, 310, 313, 315, 317, 318, 320, 324, 325, 326, 328, 347, 353, 359, 371, 377, 380, 389, 392, 395, 399, 405, 407, 409, 410, 418, 419, 421, 425, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 445, 446 and 447 with reference to amino acid positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3. Exemplary modifications include at least one amino acid replacement selected from among replacement with: histidine (H) at a position corresponding to position 1; Q at a position corresponding to position 1; E at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 15; E at a position corresponding to position 24; H at a position corresponding to position 24; E at a position corresponding to position 26; K at a position corresponding to position 26; K at a position corresponding to position 27; R at a position corresponding to position 27; E at a position corresponding to position 29; I at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; S at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; F at a position corresponding to position 37; N at a position corresponding to position 39; T at a position corresponding to position 39; R at a position corresponding to position 46; F at a position corresponding to position 48; H at a position corresponding to position 48; N at a position corresponding to position 48; Q at a position corresponding to position 52; K at a position

corresponding to position 58; Q at a position corresponding to position 58; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; R at a position corresponding to position 71; S at a position corresponding to position 71; M at a position corresponding to position 72; Q at a position corresponding to position 72; H at a position corresponding to position 73; L at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position corresponding to position 74; G at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; R at a position corresponding to position 84; A at a position corresponding to position 86; C at a position corresponding to position 87; T at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; R at a position corresponding to position 93; T at a position corresponding to position 93; R at a position corresponding to position 94; G at a position corresponding to position 97; Q at a position corresponding to position 118; F at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; H at a position corresponding to position 127; N at a position corresponding to position 127; G at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with H at a position corresponding to position 141; Y at a position corresponding to position 141; R at a position corresponding to position 142; R at a position corresponding to position 147; V at a position corresponding to position 147; K at a position corresponding to position 148; G at a position corresponding to position 150; K at a position corresponding to position 151; L at a position corresponding to position 151; ~~M at~~Mat a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; R at a position corresponding to position 152; G at a position corresponding to position 155; K at a position corresponding to position 155; D at a position corresponding to position 156; A at a position corresponding to position 163; E at a position

corresponding to position 163; K at a position corresponding to position 163; R at a position corresponding to position 163; M at a position corresponding to position 164; D at a position corresponding to position 165; N at a position corresponding to position 165; A at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; Y at a position corresponding to position 166; L at a position corresponding to position 169; R at a position corresponding to position 170; K at a position corresponding to position 174; D at a position corresponding to position 198; K at a position corresponding to position 206; L at a position corresponding to position 206; N at a position corresponding to position 212; M at a position corresponding to position 213; N at a position corresponding to position 213; M at a position corresponding to position 215; S at a position corresponding to position 219; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; R at a position corresponding to position 236; E at a position corresponding to position 237; S at a position corresponding to position 238; I at a position corresponding to position 247; T at a position corresponding to position 257; P at a position corresponding to position 259; Y at a position corresponding to position 260; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position corresponding to position 263; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; T at a position corresponding to position 272; D at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; K at a position corresponding to position 277; R at a position corresponding to position 277; T at a position corresponding to position 277; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; M at a position corresponding to position 282; V at a position corresponding to position 291; A at a position corresponding to position 293; C at a position corresponding to position 293; F at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; E at a position corresponding to position 305; G at a position corresponding to position 308; N at a position corresponding to position 308; E at a position corresponding to position 309; L at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; T at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; K at a position corresponding to position 313; R at a position corresponding to position 313; H at a position corresponding to position 315; I at a position corresponding to position 317; K at a position corresponding to position 317; R at a position corresponding to position 317; M at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position

corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 326; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; G at a position corresponding to position 347; S at a position corresponding to position 347; V at a position corresponding to position 353; with T at a position corresponding to position 359; R at a position corresponding to position 371; P at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; K at a position corresponding to position 389; M at a position corresponding to position 392; R at a position corresponding to position 395; M at a position corresponding to position 399; T at a position corresponding to position 399; W at a position corresponding to position 399; G at a position corresponding to position 405; D at a position corresponding to position 407; Q at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; P at a position corresponding to position 418; F at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; H at a position corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; K at a position corresponding to position 425; A at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; T at a position corresponding to position 438; V at a position corresponding to position 439; H at a position corresponding to position 440; R at a position corresponding to position 440; F at a position corresponding to position 441; R at a position corresponding to position 442; A at a position corresponding to position 443; M at a position corresponding to position 443; M at a position corresponding to position 445; P at a position corresponding to position 445; A at a position corresponding to position 446; D at a position corresponding to position 447; N at a position corresponding to position 447; and/or with Q at a position corresponding to position 447, with reference to amino acid positions set forth in SEQ ID NO:3.

~~[0019]~~(16) Among the polypeptides that exhibit increased hyaluronidase activity are those that exhibit at least 2.0-fold of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. For example, among these are modified PH20 polypeptides that contain at least one amino acid replacement at an amino acid position corresponding to a position selected from among 24, 29, 31, 48, 58, 69, 70, 75, 84, 97, 165, 166, 271, 278, 317, 320, 325 and 326 with reference to positions set forth in SEQ ID NO:3, wherein corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3, such as modified PH20 polypeptides that contain at least one amino acid

replacement selected from among replacement with: E at a position corresponding to position 24; E at a position corresponding to position 29; V at a position corresponding to position 31; N at a position corresponding to position 48; K at a position corresponding to position 58; Q at a position corresponding to position 58; A at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; A at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; H at a position corresponding to position 70; N at a position corresponding to position 70; R at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; L at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; G at a position corresponding to position 97; D at a position corresponding to position 165; L at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 271; H at a position corresponding to position 278; R at a position corresponding to position 278; K at a position corresponding to position 317; K at a position corresponding to position 320; E at a position corresponding to position 325, with G at a position corresponding to position 325; K at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; and V at a position corresponding to position 326; with reference to amino acid positions set forth in SEQ ID NO:3.

~~[0020]~~(17) Among any of the polypeptides provided herein that exhibit increased hyaluronidase activity, any of such modified PH20 polypeptides contain a single amino acid modification, such as a replacement, and combinations of modifications, such as at least or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 and more modifications. The modification, such as replacement, can be in an ~~the~~ unmodified PH20 polypeptide that has the sequence of amino acids set forth in SEQ ID ~~NOS~~NO: 7 or is a C-terminal truncated fragment thereof that is a soluble PH20 polypeptide, such as is set forth in any of SEQ ID NOS: 3 or 32-66, or has at least 85% sequence identity thereto. For example, any of such modified PH20 polypeptides has at least 85% sequence identity to SEQ ID NO:3.

~~[0021]~~(18) Also provided are modified PH20 polypeptides that contain at least one amino acid replacement in the PH20 polypeptide whose sequence is set forth in SEQ ID NO:7, a C-terminally truncated fragment thereof, a soluble fragment thereof, or in a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO:7, where at least one amino replacement(s) is at an amino acid position corresponding to a position selected from among 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 20, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 58, 59, 60, 61, 63, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 77, 79, 81, 82, 83, 84, 85, 86, 87, 89, 90, 91, 92, 93, 94, 96, 97, 98, 99, 102, 103, 104, 105, 106, 107, 108, 110, 114, 117, 118, 119, 120, 122, 124, 125, 127, 128, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 186, 192, 193, 195, 196, 197, 198, 200, 202, 204, 205, 206, 208, 209, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 224, 226, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 242, 245, 247, 248, 251, 253, 255, 256, 257, 258, 259,

260, 261, 263, 264, 265, 266, 267, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 297, 298, 300, 301, 302, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 320, 321, 323, 324, 325, 326, 327, 328, 331, 334, 335, 338, 339, 342, 343, 347, 348, 349, 351, 353, 356, 357, 358, 359, 360, 361, 367, 368, 369, 371, 373, 374, 375, 376, 377, 378, 379, 380, 381, 383, 385, 387, 388, 389, 391, 392, 393, 394, 395, 396, 397, 398, 399, 401, 403, 404, 405, 406, 407, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 425, 426, 427, 428, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 and 447 with reference to amino acid positions set forth in SEQ ID NO:3 or 7, where corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3; and provided that if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 13, 47, 131, or 219 the replacement is not replacement with an Alanine (A). Among these modified PH20 polypeptides are those that exhibit at least 40% of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement, where, as in all instances herein activity is compared under the same conditions.

(19) Included among these polypeptides are those that contain an amino acid replacement in the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 and 72, or in a sequence of amino acids that exhibits at least 91% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69, or 72. In particular, the modified PH20 polypeptide contains amino acid replacements in SEQ ID NO: 3, 7, 32-66, 69~~;~~, or 72, which are polypeptides that are a C-terminally truncated fragment of SEQ ID NO:7, or a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO:7. In particular, among any of such modified PH20 polypeptides provided herein are any including those in which the amino acid replacement is an amino acid replacement set forth in Table 3 below. For example, such modified PH20 polypeptides include those that have at least one amino acid replacement at an amino acid position corresponding to a position selected from among 1, 6, 8, 9, 10, 11, 12, 14, 15, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 46, 47, 48, 49, 50, 52, 58, 59, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 79, 82, 83, 84, 86, 87, 89, 90, 92, 93, 94, 97, 102, 104, 107, 114, 118, 120, 127, 128, 130, 131, 132, 135, 138, 139, 140, 141, 142, 143, 144, 146, 147, 148, 149, 150, 151, 152, 155, 156, 158, 160, 162, 163, 164, 165, 166, 167, 169, 170, 172, 173, 174, 175, 178, 179, 193, 195, 196, 198, 204, 205, 206, 209, 212, 213, 215, 219, 220, 221, 222, 232, 233, 234, 235, 236, 237, 238, 240, 247, 248, 249, 257, 258, 259, 260, 261, 263, 267, 269, 271, 272, 273, 274, 276, 277, 278, 279, 282, 283, 285, 287, 289, 291, 292, 293, 298, 305, 307, 308, 309, 310, 313, 314, 315, 317, 318, 320, 321, 324, 325, 326, 328, 335, 347, 349, 351, 353, 356, 359, 367, 368, 369, 371, 373, 374, 375, 376, 377, 380, 381, 383, 385, 389, 392, 393, 395, 396, 399, 401, 404, 405, 406, 407, 409, 410, 412, 416, 418, 419, 421, 425, 427, 428, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. Exemplary of such replacements are those that contain at least one amino acid replacement selected from among replacement with: histidine (H) at a position corresponding to position 1; A at a position corresponding to position 1; E at a position corresponding to position 1; G at a position corresponding to position 1; K at a position corresponding to position 1; Q at a position corresponding to position 1; R at a position corresponding to position 1; A at a position corresponding to position 6; M at a position corresponding to position 8; Q at a position corresponding to position 9; G at a position corresponding to position 10; H at a position corresponding to position 10; S at a position corresponding to position 11; E at a position

corresponding to position 12; I at a position corresponding to position 12; K at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 14; V at a position corresponding to position 15; M at a position corresponding to position 15; S at a position corresponding to position 20; T at a position corresponding to position 22; E at a position corresponding to position 24; H at a position corresponding to position 24; R at a position corresponding to position 24; A at a position corresponding to position 26; E at a position corresponding to position 26; K at a position corresponding to position 26; M at a position corresponding to position 26; Q at a position corresponding to position 26; R at a position corresponding to position 26; D at a position corresponding to position 27; K at a position corresponding to position 27; R at a position corresponding to position 27; R at a position corresponding to position 28; E at a position corresponding to position 29; I at a position corresponding to position 29; K at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; R at a position corresponding to position 29; S at a position corresponding to position 29; T at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; L at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; G at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; W at a position corresponding to position 31; C at a position corresponding to position 32; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; G at a position corresponding to position 33; W at a position corresponding to position 34; Q at a position corresponding to position 35; V at a position corresponding to position 35; H at a position corresponding to position 36; N at a position corresponding to position 36; F at a position corresponding to position 37; M at a position corresponding to position 37; Y at a position corresponding to position 38; A at a position corresponding to position 39; L at a position corresponding to position 39; N at a position corresponding to position 39; T at a position corresponding to position 39; L at a position corresponding to position 40; T at a position corresponding to position 41; L at a position corresponding to position 46; R at a position corresponding to position 46; D at a position corresponding to position 47; F at a position corresponding to position 47; T at a position corresponding to position 47; W at a position corresponding to position 47, with F at a position corresponding to position 48; H at a position corresponding to position 48; K at a position corresponding to position 48; N at a position corresponding to position 48; R at a position corresponding to position 49; D at a position corresponding to position 50; S at a position corresponding to position 50; M at a position corresponding to position 50; N at a position corresponding to position 52; Q at a position corresponding to position 52; R at a position corresponding to position 52; S at a position corresponding to position 52; T at a position corresponding to position 52; C at a position corresponding to position 58; K at a position

corresponding to position 58; L at a position corresponding to position 58; P at a position corresponding to position 58; Q at a position corresponding to position 58; R at a position corresponding to position 58; H at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; N at a position corresponding to position 59; K at a position corresponding to position 63; L at a position corresponding to position 63; M at a position corresponding to position 63; R at a position corresponding to position 63; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; P at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; E at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; T at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; Y at a position corresponding to position 70; G at a position corresponding to position 71; N at a position corresponding to position 71; R at a position corresponding to position 71; S at a position corresponding to position 71; K at a position corresponding to position 72; M at a position corresponding to position 72; Q at a position corresponding to position 72; A at a position corresponding to position 73; H at a position corresponding to position 73; K at a position corresponding to position 73; L at a position corresponding to position 73; Q at a position corresponding to position 73; R at a position corresponding to position 73; T at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position corresponding to position 74; E at a position corresponding to position 74; F at a position corresponding to position 74; G at a position corresponding to position 74; H at a position corresponding to position 74; K at a position corresponding to position 74; L at a position corresponding to position 74; M at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; M at position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; L at a position corresponding to position 79; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; E at a position corresponding to position 84; F at a position corresponding to position 84; G at a position corresponding to position 84; N at a position corresponding to position 84; R at a position corresponding to position 84; A at a position

corresponding to position 86; H at a position corresponding to position 86; K at a position corresponding to position 86; N at a position corresponding to position 86; S at a position corresponding to position 86; T at a position corresponding to position 86; W at a position corresponding to position 86; C at a position corresponding to position 87; G at a position corresponding to position 87; L at a position corresponding to position 87; M at a position corresponding to position 87; R at a position corresponding to position 87; S at a position corresponding to position 87; T at a position corresponding to position 87; V at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 89; A at a position corresponding to position 90; E at a position corresponding to position 90; H at a position corresponding to position 90; K at a position corresponding to position 90; N at a position corresponding to position 90; R at a position corresponding to position 90; C at a position corresponding to position 92; L at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; Q at a position corresponding to position 93; R at a position corresponding to position 93; S at a position corresponding to position 93; T at a position corresponding to position 93; D at a position corresponding to position 94; Q at a position corresponding to position 94; R at a position corresponding to position 94; A at a position corresponding to position 97; C at an amino acid residue corresponding to position 97; D at a position corresponding to position 97; E at a position corresponding to position 97; G at a position corresponding to position 97; L at a position corresponding to position 97; S at a position corresponding to position 97; S at a position corresponding to position 102; T at a position corresponding to position 102; R at a position corresponding to position 104; L at a position corresponding to position 107; A at a position corresponding to position 114; Q at a position corresponding to position 118; H at a position corresponding to position 120; F at a position corresponding to position 120; I at a position corresponding to position 120; S at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; E at a position corresponding to position 127; H at a position corresponding to position 127; N at a position corresponding to position 127; Q at a position corresponding to position 127; R at a position corresponding to position 127; I at a position corresponding to position 128; R at a position corresponding to position 130; G at a position corresponding to position 131; I at a position corresponding to position 131; M at a position corresponding to position 131; Q at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; N at a position corresponding to position 132; L at a position corresponding to position 132; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with L at a position corresponding to position 138; T at a position corresponding to position 139; K at a position corresponding to position 140; H at a position corresponding to position 141; R at a position corresponding to position 141; S at a position corresponding to position 141; W at a position corresponding to position 141; Y at a position corresponding to position 141; D at a position corresponding to position 142; G at a position corresponding to position 142; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; Q at a position corresponding to position 142; R at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; R at a position corresponding to position 144; T at a position corresponding to position 144; P at a

position corresponding to position 146; R at a position corresponding to position 146; A at a position corresponding to position 147; F at a position corresponding to position 147; L at a position corresponding to position 147; R at a position corresponding to position 147; S at a position corresponding to position 147; V at a position corresponding to position 147; H at a position corresponding to position 148; K at a position corresponding to position 148; Q at a position corresponding to position 148; T at a position corresponding to position 149; V at a position corresponding to position 149; A at a position corresponding to position 150; D at a position corresponding to position 150; G at a position corresponding to position 150; N at a position corresponding to position 150; S at a position corresponding to position 150; W at a position corresponding to position 150; Y at a position corresponding to position 150; A at a position corresponding to position 151; H at a position corresponding to position 151; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; S at a position corresponding to position 151; T at a position corresponding to position 151; V at a position corresponding to position 151; W at a position corresponding to position 151; Y at a position corresponding to position 151; R at a position corresponding to position 152; T at a position corresponding to position 152; W at a position corresponding to position 152; D at a position corresponding to position 155; G at a position corresponding to position 155; K at a position corresponding to position 155; R at a position corresponding to position 155; D at a

position corresponding to position 156; Q at a position corresponding to position 158; S at a position corresponding to position 158; S at a position corresponding to position 160; E at a position corresponding to position 162; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; Q at a position corresponding to position 163; R at a position corresponding to position 163; S at a position corresponding to position 163; M at a position corresponding to position 164; V at a position corresponding to position 164; D at a position corresponding to position 165; F at a position corresponding to position 165; N at a position corresponding to position 165; S at a position corresponding to position 165; V at a position corresponding to position 165; A at a position corresponding to position 166; E at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; W at a position corresponding to position 166; Y at a position corresponding to position 166; D at a position corresponding to position 167; L at a position corresponding to position 169; R at a position corresponding to position 170; A at a position corresponding to position 172; R at a position corresponding to position 173; G at a position corresponding to position 174; K at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 174; T at a position corresponding to position 174; T at a position corresponding to position 175; K at a position corresponding to position 178; R at a position corresponding to position 178; K at a position corresponding to position 179; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; with E at a position corresponding to position 196; R at a position corresponding to position 196; with D at a position corresponding to position 198; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; L at a position corresponding to position 205; T at a

position corresponding to position 205; I at a position corresponding to position 206; K at a position corresponding to position 206; L at a position corresponding to position 206; R at a position corresponding to position 206; R at a position corresponding to position 209; N at a position corresponding to position 212; S at a position corresponding to position 212; A at a position corresponding to position 213; M at a position corresponding to position 213; N at a position corresponding to position 213; H at a position corresponding to position 215; M at a position corresponding to position 215; I at a position corresponding to position 219; K at a position corresponding to position 219; S at a position corresponding to position 219; H at a position corresponding to position 220; I at a position corresponding to position 220; L at a position corresponding to position 220; V at a position corresponding to position 220; Q at a position corresponding to position 221; G at a position corresponding to position 222; F at a position corresponding to position 232; G at a position corresponding to position 233; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; A at a position corresponding to position 235; R at a position corresponding to position 236; C at a position corresponding to position 237; E at a position corresponding to position 237; H at a position corresponding to position 237; Q at a position corresponding to position 237; T at a position corresponding to position 237; E at a position corresponding to position 238; H at a position corresponding to amino acid position 238; S at a position corresponding to position 238; A at a position corresponding to position 240; Q at a position corresponding to position 240; I at a position corresponding to position 247; A at a position corresponding to position 248; V at a position corresponding to position 249; G at a position corresponding to position 257; T at a position corresponding to position 257; R at a position corresponding to position 257; N at a position corresponding to position 258; S at a position corresponding to position 258; P at a position corresponding to position 259; M at a position corresponding to position 260; Y at a position corresponding to position 260; A at a position corresponding to position 261; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position corresponding to position 263; T at a position corresponding to position 267; A at a position corresponding to position 269; L at a position corresponding to position 271; ~~M at~~Mat a position corresponding to position 271; D at a position corresponding to position 272; T at a position corresponding to position 272; H at a position corresponding to position 273; Y at a position corresponding to position 273; F at a position corresponding to position 274; D at a position corresponding to position 276; H at a position corresponding to position 276; M at a position corresponding to position 276; R at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; A at a position corresponding to position 277; E at a position corresponding to position 277; H at a position corresponding to position 277; K at a position corresponding to position 277; M at a position corresponding to position 277; N at a position corresponding to position 277; Q at a position corresponding to position 277; R at a position corresponding to position 277; S at a position corresponding to position 277; T at a position corresponding to position 277; E at a position corresponding to position 278; F at a position corresponding to position 278; G at a position corresponding to position 278; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; H at a position corresponding to position 279; M at a position corresponding to position 282; S at a

position corresponding to position 283; H at a position corresponding to position 285; T at a position corresponding to position 287; S at a position corresponding to position 289; S at a position corresponding to position 291; V at a position corresponding to position 291; C at a position corresponding to position 292; F at a position corresponding to position 292; H at a position corresponding to position 292; K at a position corresponding to position 292; R at a position corresponding to position 292; V at a position corresponding to position 292; A at a position corresponding to position 293; C at a position corresponding to position 293; D at a position corresponding to position 293; F at a position corresponding to position 293; K at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; Y at a position corresponding to position 293; G at a position corresponding to position 298; E at a position corresponding to position 305; G at a position corresponding to position 307; D at a position corresponding to position 308; G at a position corresponding to position 308; K at a position corresponding to position 308; N at a position corresponding to position 308; R at a position corresponding to position 308; E at a position corresponding to position 309; G at a position corresponding to position 309; H at a position corresponding to position 309; L at a position corresponding to position 309; M at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; S at a position corresponding to position 309; T at a position corresponding to position 309; V at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; Q at a position corresponding to position 310; S at a position corresponding to position 310; A at a position corresponding to position 313; G at a position corresponding to position 313; H at a position corresponding to position 313; K at a position corresponding to position 313; P at a position corresponding to position 313; R at a position corresponding to position 313; T at a position corresponding to position 313; Y at a position corresponding to position 313; with S at a position corresponding to position 314; Y at a position corresponding to position 314; A at a position corresponding to position 315; H at a position corresponding to position 315; Y at a position corresponding to position 315; A at a position corresponding to position 317; I at a position corresponding to position 317; K at a position corresponding to position 317; N at a position corresponding to position 317; Q at a position corresponding to position 317; R at a position corresponding to position 317; S at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; H at a position corresponding to position 318; K at a position corresponding to position 318; M at a position corresponding to position 318; R at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 321; S at a position corresponding to position 321; N at a position corresponding to position 324; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 325; L at a position corresponding to position 326; V at a position corresponding to position 326; C at a

position corresponding to position 328; G at a position corresponding to position 328; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; S at a position corresponding to position 335; A at a position corresponding to position 347; G at a position corresponding to position 347; S at a position corresponding to position 347; M at a position corresponding to position 349; R at a position corresponding to position 349; S at a position corresponding to position 351; V at a position corresponding to position 353; with H at a position corresponding to position 356; S at a position corresponding to position 356; E at a position corresponding to position 359; H at a position corresponding to position 359; T at a position corresponding to position 359; A at a position corresponding to position 367; G at a position corresponding to position 367; K at a position corresponding to position 367; S at a position corresponding to position 367; A at a position corresponding to position 368; E at a position corresponding to position 368; K at a position corresponding to position 368; L at a position corresponding to amino acid position 368; M at a position corresponding to amino acid position 368; R at a position corresponding to position 368; T at a position corresponding to amino acid position 368; H at a position corresponding to position 369; R at a position corresponding to position 369; F at a position corresponding to position 371; H at a position corresponding to position 371; K at a position corresponding to position 371; L at a position corresponding to position 371; R at a position corresponding to position 371; S at a position corresponding to position 371; M at a position corresponding to position 373; H at a position corresponding to position 374; P at a position corresponding to position 374; A at a position corresponding to position 375; G at a position corresponding to position 375; K at a position corresponding to position 375; R at a position corresponding to position 375; D at a position corresponding to position 376; E at a position corresponding to position 376; Q at a position corresponding to position 376; R at a position corresponding to position 376; T at a position corresponding to position 376; V at a position corresponding to position 376; Y at a position corresponding to position 376; D at a position corresponding to position 377; E at a position corresponding to position 377; H at a position corresponding to position 377; K at a position corresponding to position 377; P at a position corresponding to position 377; R at a position corresponding to position 377; S at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; S at a position corresponding to position 381; I at a position corresponding to position 383; K at a position corresponding to position 383; L at a position corresponding to position 383; S at a position corresponding to position 383; A at a position corresponding to position 385; Q at a position corresponding to position 385; V at a position corresponding to position 385; A at a position corresponding to position 389; G at a position corresponding to position 389; L at a position corresponding to position 389; K at a position corresponding to position 389; Q at a position corresponding to position 389; S at a position corresponding to position 389; A at a position corresponding to position 392; F at a position corresponding to position 392; M at a position corresponding to position 392; Q at a position corresponding to position 392; R at a position corresponding to position 392; V at a position corresponding to position 392; F at a position corresponding to position 393; M at a position corresponding to position 393; A at a position corresponding to position 395; H at a position corresponding to position 395; R at a position corresponding to position 395; A at a position corresponding to position 396; H at a position corresponding to position 396; Q at a position corresponding to position 396; S at a position

corresponding to position 396; K at a position corresponding to position 399; M at a position corresponding to position 399; T at a position corresponding to position 399; V at a position corresponding to position 399; W at a position corresponding to position 399; A at a position corresponding to position 401; E at a position corresponding to position 401; A at a position corresponding to position 404; G at a position corresponding to position 405; F at a position corresponding to position 406; N at a position corresponding to position 406; A at a position corresponding to position 407; D at a position corresponding to position 407; E at a position corresponding to position 407; F at a position corresponding to position 407; H at a position corresponding to position 407; Q at a position corresponding to position 407; P at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; Q at a position corresponding to position 412; R at a position corresponding to position 412; V at a position corresponding to position 412; L at a position corresponding to position 416; E at a position corresponding to position 418; L at a position corresponding to position 418; P at a position corresponding to position 418; R at a position corresponding to position 418; V at a position corresponding to position 418; F at a position corresponding to position 419; H at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; Y at a position corresponding to position 419; A at a position corresponding to position 421; H at a position corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; G at a position corresponding to position 425; K at a position corresponding to position 425; Q at a position corresponding to position 427; T at a position corresponding to position 427; L at a position corresponding to position 428; A at a position corresponding to position 431; G at a position corresponding to position 431; E at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; L at a position corresponding to position 431; N at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; A at a position corresponding to position 433; H at a position corresponding to position 433; I at a position corresponding to position 433; K at a position corresponding to position 433; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; W at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; A at a position corresponding to position 438; D at a position corresponding to position 438; E at a position corresponding to position 438; L at a position corresponding to position 438; N at a position corresponding to position 438; T at a position corresponding to position 438; A at a position corresponding to position 439; C at a position corresponding to position 439; K at a position corresponding to position 439; P at a position corresponding to position 439; Q at a position corresponding to position 439; T at a position corresponding to position 439; V at a position corresponding to position 439; D at a position corresponding to position 440; H at a position corresponding to position 440; M at a position corresponding to position 440; P at a position corresponding to position 440; R at a position corresponding to position 440; S at a position corresponding to position 440; A at a position

corresponding to position 441; F at a position corresponding to position 441; C at a position corresponding to position 442; G at a position corresponding to position 442; R at a position corresponding to position 442; A at a position corresponding to position 443; E at a position corresponding to position 443; F at a position corresponding to position 443; G at a position corresponding to position 443; M at a position corresponding to position 443; N at a position corresponding to position 443; E at a position corresponding to position 444; H at a position corresponding to position 444; V at a position corresponding to position 444; H at a position corresponding to position 445; M at a position corresponding to position 445; N at a position corresponding to position 445; P at a position corresponding to position 445; Q at a position corresponding to position 445; S at a position corresponding to position 445; T at a position corresponding to position 445; V at a position corresponding to position 445; W at a position corresponding to position 445; A at a position corresponding to position 446; M at a position corresponding to position 446; W at a position corresponding to position 446; D at a position corresponding to position 447; E at a position corresponding to position 447; G at a position corresponding to position 447; I at a position corresponding to position 447; N at a position corresponding to position 447; P at a position corresponding to position 447; Q at a position corresponding to position 447; T at a position corresponding to position 447, and/or replacement with V at a position corresponding to position 447, each with reference to amino acid positions set forth in SEQ ID NO:3. Among these modified PH20 polypeptides are those that exhibit at least 40% of the activity of the PH20 that does not contain the particular amino acid replacement. Activity can vary between, for example, 40% to 5000%, 40% to 2000%, 40% to 1000%, 40% to 500%, 40% to 100%, 80% to 2000%, 80% to 600%, 80% to 200%, 80% to 300%, of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. Such activity includes at least 50%, 60%, 70%, 80%, 90%, 100%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, 2000%, 3000% or more of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement, where, as in all instances herein, the activities are compared under the same conditions.

~~[0023]~~(20) In particular, provided are modified PH20 polypeptides that contain at least one amino acid replacement in a PH20 polypeptide set forth in SEQ ID NO:7, a C-terminally truncated fragment thereof, or in a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO:7 or a corresponding truncated fragment, where: the modified PH20 polypeptides exhibit less than 20% of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement, where activities are compared under the same conditions; the amino acid replacement(s) is at an amino acid position corresponding to a position selected from among 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 27, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 94, 95, 96, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 143, 144, 145, 149, 150, 152, 153, 154, 155, 156, 157, 158, 159, 161, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 197, 198, 199, 200, 201, 202, 203, 204, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 238, 239, 240, 241, 242, 243, 244, 245,

246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290~~;~~, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 331, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 408, 410, 411, 412, 413, 414, 415, 416, 417, 419, 420, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 434, 437, 438, 439, 440, 441, 442, 443, 444, or 447 with reference to amino acid positions set forth in SEQ ID NO:3 or 7;

(21) corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide set forth in SEQ ID NO:3; and provided that:~~[0024]~~ (i) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 200, 333, 358 or 393 the replacement is not replacement with an Alanine (A).~~[0025]~~ (ii) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 111 or 249 the replacement is not replacement with an asparagine (N);~~[0026]~~ (iii) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 113 the replacement is not replacement with a glutamine (Q);~~[0027]~~ (iv) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 176 the replacement is not replacement with a glycine (G); and~~[0028]~~ (v) if the modified PH20 polypeptide contains an amino acid replacement at a position corresponding to position 252 the replacement is not replacement with a threonine (T).

~~[0029]~~(22) Exemplary of such modified PH20 polypeptides are any that contain amino acid replacement(s) in a PH20 polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 32-66, 69, or 72, or in a sequence of amino acids that exhibits at least 91% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69, or 72. For example, the modified PH20 polypeptide contains amino acid replacement(s) in SEQ ID NOS: 3, 7, 32-66, 69, or 72, which are polypeptides that are a C-terminally truncated fragment of SEQ ID NO:7, or a PH20 polypeptide that has a sequence of amino acids that is at least 91% identical to the sequence of amino acids set forth in SEQ ID NO:7. In examples of such modified PH20 polypeptides provided herein, the modified PH20 polypeptides can exhibit similar or the same activity as the PH20 without the modification, or can exhibit increased activity or activity that is less than 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.05% or less of the hyaluronidase activity of the PH20 polypeptide not containing the amino acid replacement. Exemplary of such modified PH20 polypeptides are any set forth in Table 5.

~~[0030]~~(23) Among any and all of the modified PH20 polypeptides provided herein and above, the modified PH20 polypeptide is one that does not consist of the sequence of amino acids set forth in any of SEQ ID NOS: 3, 6-66, 69-72, 856-861, 869 or 870. In particular, among any of the modified PH20 polypeptides provided herein above or elsewhere herein are any that contain an amino acid replacement(s) in a PH20 polypeptide having the sequence of amino acids set forth any of SEQ ID NO: 3, 7, 69 or 72 provided that: (i) where the modified PH20 polypeptide includes only a single amino acid replacement the replacement does not corresponds to amino

acid replacements V12A, N47A, D111N, E113Q, N131A, R176G, N200A, N219A, E249Q, R252T, N333A or N358A, with reference to amino acid positions set forth in SEQ ID NO:3; (ii) where the modified PH20 polypeptide includes only two amino acid replacements the replacements do not correspond to amino acid replacements P13A/L464W, N47A/N131A, N47A/N219A, N131A/N219A or N333A/N358A with reference to positions set forth in SEQ ID NO:3; and (iii) where the modified PH20 polypeptide includes only three amino acid replacements the replacements ~~does~~do not correspond to amino acid replacements N47A/N131A/N219A, with reference to amino acid positions set forth in SEQ ID NO:3.

~~[0031]~~(24) Any of the above modified PH20 polypeptides and any provided herein and described above and below can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, or more of the amino acid replacements. The modified PH20 polypeptides can include a signal sequence, including the native sequence or a heterologous sequence or a modified sequence, and also include a mature PH20 polypeptide that lacks the signal sequence.

~~[0032]~~(25) Among any of the modified PH20 polypeptides provided herein above or described below are modified PH20 polypeptides that contain or have the sequence of amino acids set forth in any of SEQ ID NOS: 73-855 or a sequence of amino acids that exhibits at least 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a sequence of amino acids set forth in any of SEQ ID NOS: 73-855 and that contains at least one amino acid replacement, such as any described above or elsewhere herein, with reference to positions compared to the sequence of amino acids set forth in SEQ ID NO:3. In any of the examples of the modified PH20 polypeptides provided herein, the modified PH20 polypeptide does not have or contain the sequence of amino acids set forth in any of SEQ ID NOS: 8-31, 69-72, 856-861, 869 or 870.

~~[0033]~~(26) The modified PH20 polypeptides provided herein can be substantially purified or isolated, can exhibit catalytic activity at neutral pH, can be secreted upon expression from cells and are soluble in the supernatant, and/or can include modified amino acids, such as a modification selected from among glycosylation, sialation, albumination, farnysylation, carboxylation, hydroxylation, conjugation to a polymer, such as PEGylation or conjugation to dextran, conjugation to another moiety, such as a multimerization domain, toxin, detectable label or drug, and phosphorylation. The modified PH20 polypeptide can be glycosylated, such as by containing at least an N-acetylglucosamine moiety linked to each of at least three asparagine (N) residues, where, for example, the three asparagine residues correspond to amino acid residues 200, 333 and 358 of SEQ ID NO:3. Multimerization domains include Fc domains.

~~[0034]~~(27) Also provided are nucleic acid molecules that encode any of the modified PH20 polypeptides provided herein. Vectors, eukaryotic and prokaryotic, that contain the nucleic acid molecules are provided. The vectors include expression vectors and include mammalian vectors, including viral vectors. Viral vectors include adenovirus vectors, retrovirus vectors, vaccinia virus vectors, herpes simplex virus and cytomegalovirus vectors, and other such viral vectors. Of interest are oncolytic vectors that accumulate in or are targeted to tumors. Also provided are cells that contain the nucleic acid molecules and cells that contain the vectors. The cells can be

prokaryotic or eukaryotic, particularly mammalian cells, such as Chinese Hamster Ovary (CHO) cells.

[0035](28) Also provided herein is a modified PH20 polypeptide that is produced by any of the provided cells. Thus, provided herein are methods of producing a modified PH20 polypeptide by culturing any of the cells provided herein under conditions whereby an encoded modified PH20 polypeptide is produced and secreted by the cell, and recovering the expressed polypeptide. Also provided herein is a method of producing a modified PH20 polypeptide by introducing any of the nucleic acids provided herein or any of the vectors provided herein into a cell capable of incorporating N-linked sugar moieties into the polypeptide, culturing the cell under conditions whereby an encoded modified PH20 polypeptide is produced and secreted by the cell, and recovering the expressed polypeptide. In such examples, the nucleic acid is operably linked to a promoter. The cultured cell can be a eukaryotic cell, such as a mammalian cell, for example, a Chinese hamster ovary (CHO) cell.

[0036](29) Also provided are pharmaceutical compositions that contain any of the modified PH20 polypeptides provided herein or any of the nucleic acids or vectors provided herein. The compositions can be formulated with other agents and/or with other components, such as preservatives. The compositions can be formulated so that the components, particularly the PH20 and any other active agent, remain active or are stable under preselected conditions. In addition, as described herein, the PH20 polypeptides are modified so that they exhibit increased stability under various conditions. For example, provided are compositions in which the modified PH20 polypeptide is stable (i.e., retains activity as described herein) at a temperature from or from about 2.degree.° C. to 8.degree.° C., inclusive, for at least 1 month or is stable at a temperature from or from about 30.degree.° C. to 42.degree.° C., inclusive, for at least 3 days. Provided are compositions in which the modified PH20 polypeptide in the composition is stable at a temperature from or from about 2.degree.° C. to 8.degree.° C., inclusive, for at least 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23 months, 24 months, 25 months, 26 months, 27 months, 28 months, 29 months or 30 months. Also provided are compositions in which the modified PH20 polypeptide in the composition is stable at a temperature from or from about 30.degree.° C. to 42.degree.° C., inclusive, for at least 3 days, at least 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 35 days, 40 days, 45 days, 50 days, 60 days or more. The pharmaceutical compositions can contain a pharmaceutically acceptable excipient.

[0037](30) The conditions, formulations, components, and modified PH20 polypeptide are chosen to achieve a desired stability. The pharmaceutical compositions can be formulated for direct administration or can require dilution. They can be formulated for multiple or single dosage administration. Exemplary compositions include concentrations of modified PH20 between or about between 0.1 .mu.gμg/mL to 100 .mu.gμg/mL, 1 .mu.gμg/mL to 50 .mu.gμg/mL or 1 .mu.gμg/mL to 20 .mu.gμg/mL, or 10 U/mL to 5000 U/mL, 50 U/mL to 4000 U/mL, 100 U/mL to 2000 U/mL, 300 U/mL to 2000 U/mL, 600 U/mL to 2000 U/mL, or 100 U/mL to 1000 U/mL. Exemplary salts include NaCl at a concentration, for example, of less than or about or 200 mM, 180 mM, 150 mM, 140 mM, 130 mM, 120 mM, 110 mM, 100 mM, 90 mM, 80 mM, 70

mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less, or between or about between 0.1 mM to 200 mM, 0.1 mM to 100 mM, 120 mM to 200 mM, 10 mM to 50 mM, 10 mM to 90 mM, 80 mM to 200 mM, 80 mM to 140 mM, 50 mM to 100 mM, 80 mM to 100 mM, 50 mM to 80 mM, 100 mM to 140 mM or 120 mM to 140 mM.

~~[0038]~~(31) The pharmaceutical compositions can contain an anti-microbially effective amount of a preservative or mixture of preservatives, such as one, two, three, four or more of a phenolic preservative(s), a non-phenolic preservative(s) or a phenolic preservative(s) and a non-phenolic preservative(s), such as, but not limited to, phenol, m-cresol, methylparaben, benzyl alcohol, thimerosal, benzalkonium chloride, 4-chloro-1-butanol, chlorhexidine dihydrochloride, chlorhexidine digluconate, L-phenylalanine, EDTA, bronopol, phenylmercuric acetate, glycerol, imidurea, chlorhexidine, sodium dehydroacetate, o-cresol, p-cresol, chlorocresol, cetrimide, benzethonium chloride, ethyl paraben, propylparaben, butylparaben and any combinations thereof. Phenols include, for example, phenol, metacresol (m-cresol), benzyl alcohol, and parabens, such as methylparaben or propylparaben. Anti-microbial effective concentrations of one or more preservative agents (as a percentage (%) of mass concentration (w/v)) can be between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive. Examples thereof are pharmaceutical compositions where the preservatives are phenol, m-cresol or phenol and m-cresol and the amount as a % of mass concentration (w/v) in the formulation is between or about between 0.1% to 0.25% phenol and between or about between 0.05% to 0.2% m-cresol, is between or about between 0.10% to 0.2% phenol and between or about between 0.6% to 01.8% m-cresol, between or about between 0.1% to 0.15% phenol and 0.8% t 0.15% m-cresol, is between or about between 0.10% to 0.15% phenol and between or about between 0.06 to 0.09% m-cresol or is between or about between 0.12% to 0.18% phenol and between or about between 0.14 to 0.22% m-cresol.

~~[0039]~~(32) The pharmaceutical compositions can contain a further therapeutically active agent. The active agent can be formulated in the composition or provided as a combination with the PH20-containing composition, but in a separate composition for administration separately, sequentially, intermittently, simultaneously or together. Therapeutically active agents include, for example, an agent selected from among a chemotherapeutic agent, an analgesic agent, an anti-inflammatory agent, an antimicrobial agent, an amoebicidal agent, a trichomonacidal agent, an anti-parkinson agent, an anti-malarial agent, an anticonvulsant agent, an anti-depressant agent, and antiarthritics agent, an anti-fungal agent, an antihypertensive agent, an antipyretic agent, an anti-parasite agent, an antihistamine agent, an alpha-adrenergic agonist agent, an alpha blocker agent, an anesthetic agent, a bronchial dilator agent, a biocide agent, a bactericide agent, a bacteriostat agent, a beta adrenergic blocker agent, a calcium channel blocker agent, a cardiovascular drug agent, a contraceptive agent, a decongestant agent, a diuretic agent, a depressant agent, a diagnostic agent, a electrolyte agent, a hypnotic agent, a hormone agent, a hyperglycemic agent, a muscle relaxant agent, a muscle contractant agent, an ophthalmic agent, a parasympathomimetic agent, a psychic energizer agent, a sedative agent, a sympathomimetic agent, a tranquilizer agent, an urinary agent, a vaginal agent, a viricide agent, a vitamin agent, a non-steroidal anti-inflammatory agent, an angiotensin converting enzyme inhibitor agent, a polypeptide, a protein, a nucleic acid, a drug, an organic molecule and a sleep inducer. Exemplary of such agents are antibodies, particularly monoclonal antibodies, an Immune Globulin preparation, a bisphosphonate, a cytokine, a chemotherapeutic agent, a coagulation

factor and an insulin. Insulins include, for example, basal insulins and fast-acting insulin, such as regular insulin, particularly recombinant human insulin, and insulin analogs, such as insulin lispro, insulin aspart or insulin glulisine. Particular fast-acting insulins are those with an A chain having a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in SEQ ID NO:863 or an insulin with an A chain with a sequence of amino acids set forth as amino acid residue positions 88-108 of SEQ ID NO:864 and a B chain with a sequence of amino acids set forth as amino acid residue positions 25-54 of SEQ ID NO:864 or an insulin analog that is selected from among an insulin having an A chain with a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in any of SEQ NOS:865-867. The amount of fast-acting insulin in the compositions can be empirically determined, but typically can be 10 U/mL to 1000 U/mL, 50 U/mL to 500 U/mL, 100 U/mL to 1000 U/mL or 500 U/mL to 1000 U/mL, inclusive.

~~[0040]~~(33) In particular examples, provided herein is a pharmaceutical composition containing any of the modified PH20 polypeptides provided herein that exhibit increased stability to a phenolic preservative and an insulin, such as a fast-acting insulin. The modified PH20 polypeptides ~~an~~and insulin can be provided in therapeutically effective amounts. For example, provided herein is a pharmaceutical composition ~~contain~~that contains any of the modified PH20 ~~polypeptide~~polypeptides provided herein that exhibits increased stability to a phenolic preservative in an ~~mount~~amount that is from or from about 100 U/mL to 1000 U/mL and a fast-acting insulin in an amount that is from or from about 10 U/mL to 1000 U/mL. For example, the fast-acting insulin can be an insulin analog, such as insulin lispro, insulin aspart or insulin glulisine or other analog. Any of such pharmaceutical compositions can be formulated at a pH that is from or from about 7.0 to 7.6. Any of such pharmaceutical compositions also can be formulated to contain salt, such as NaCl, at a concentration that is from or from about 0.1 mM to 200 mM and/or an anti-microbial ~~effect~~effective amount of at least one preservative where the composition generally contains at least one phenolic preservative. The anti-microbial effective amount is a total amount of one or more preservative agents as a percentage (%) of mass concentration (w/v) that is or is between 0.05% to 0.6%. The phenolic preservative(s) ~~is(are)~~ can be a phenol, metacresol (m-cresol), benzyl alcohol, ~~and~~or a paraben. In any of the above examples of a pharmaceutical composition, the composition also can contain a surfactant, such as a polypropylene glycol, polyethylene glycol, glycerin, sorbitol, poloxamer or polysorbate, in an amount as a % of mass concentration (w/v) in the formulation that is at least or at least about 0.001%; a buffering agent that is a non-metal binding agent or is a metal binding agent, such as Tris, histidine, phosphate or citrate, wherein the concentration of the buffering agent is between or between about 1 mM to 100 mM; glycerin in a concentration less than 60 mM; an antioxidant, such as cysteine, tryptophan or methionine, at a concentration between or from about between 2 mM to 50 mM, inclusive; and/or zinc at a concentration of between or about between 0.001 to 0.1 mg per 100 units of insulin (mg/100 U). Also provided herein are closed loop systems, insulin pumps including continuous subcutaneous infusion insulin (CSII) pumps and insulin pens that contain any of the pharmaceutical compositions. The pharmaceutical compositions can be used in methods or uses for treating diabetes, such as type 1 diabetes mellitus, type 2 diabetes mellitus or gestational diabetes.

~~[0041]~~(34) Other therapeutic agents in any of the pharmaceutical compositions provided herein include, but are not limited to Adalimumabs, Agalsidase Betas, Alefacepts, Ampicillins, Anakinras, Antipoliomyelitic Vaccines, Anti-Thymocytes, Azithromycins, Becaplermins,

Caspofungins, Cefazolins, Cefepimes, Cefotetans, Ceftazidimes, Ceftriaxones, Cetuximabs, Cilastatins, Clavulanic Acids, Clindamycins, Darbepoetin Alfas, Daclizumabs, Diphtheria, Diphtheria antitoxins, Diphtheria Toxoids, Efalizumabs, Epinephrines, Erythropoietin Alphas, Etanercepts, Filgrastims, Fluconazoles, Follicle-Stimulating Hormones, Follitropin Alphas, Follitropin Betas, ~~Fosphenyloins~~Fosphenytoins, Gadodiamides, Gadopentetates, Gatifloxacins, Glatiramers, GM-CSF's, Goserelins, Goserelin acetates, Granisetrons, Haemophilus Influenza B's, Haloperidols, Hepatitis vaccines, Hepatitis A Vaccines, Hepatitis B Vaccines, Ibritumomab Tiuxetans, Ibritumomabs, Tiuxetans, Immunoglobulins, Hemophilus influenza vaccines, Influenza Virus Vaccines, Infliximabs, Insulin lispro, 75% neutral protamine lispro (NPL)/25% insulin lispro, 50% neutral protamine Hagedorn (NPH)/50% regular insulin, 70% NPH/30% regular insulin; Regular insulin, NPH insulin, Ultra insulin, Ultralente insulin, and Insulin Glargines, Interferons, Interferon alpha, Interferon Betas, Interferon Gammas, Interferon alpha-2a, Interferon alpha 2-b, Interferon Alphacon, Interferon alpha-n, Interferon Betas, Interferon Beta-1a's, Interferon Gammas, Interferon alpha-con, Iodixanols, ~~Iohexyls~~Iohexols, Iopamidols, Ioversols, Ketorolacs, Laronidases, Levofloxacins, Lidocaines, Linezolids, Lorazepams, Measles Vaccines, Measles virus, Mumps viruses, Measles-Mumps-Rubella Virus Vaccines, Rubella vaccines, Medroxyprogesterones, Meropenems, Methylprednisolones, Midazolams, Morphines, Octreotides, Omalizumabs, Ondansetrons, Palivizumabs, Pantoprazoles, Pegaspargases, Pegfilgrastims, Peg-Interferon Alpha-2a's, Peg-Interferon Alpha-2b's, Pegvisomants, Pertussis vaccines, Piperacillins, Pneumococcal Vaccines and Pneumococcal Conjugate Vaccines, Promethazines, Reteplases, Somatropins, Sulbactams, Sumatriptans, Tazobactams, Tenecteplases, Tetanus Purified Toxoids, Ticarcillins, Tositumomabs, Triamcinolones, Triamcinolone Acetonides, Triamcinolone hexacetonides, Vancomycins, Varicella Zoster immunoglobulins, Varicella vaccines, other vaccines, Alemtuzumabs, Alitretinoins, Allopurinols, Altretamines, Amifostines, Anastrozoles, Arsenics, Arsenic Trioxides, Asparaginases, Bacillus Calmette-Guerin (BCG) vaccines, BCG Live, Bexarotenes, Bleomycins, Busulfans, Busulfan intravenous, Busulfan orals, Calusterones, Capecitabines, Carboplatins, Carmustines, Carmustines with Polifeprosans, Celecoxibs, Chlorambucils, Cisplatins, Cladribines, Cyclophosphamides, Cytarabines, Cytarabine liposomals, Dacarbazines, Dactinomycins, Daunorubicin liposomals, Daunorubicins, Daunomycins, Denileukin Diftitoxes, Dexrazoxanes, Docetaxels, Doxorubicins, Doxorubicin liposomals, Dromostanolone propionates, Elliott's B Solutions, Epirubicins, Epoetin alfas, Estramustines, Etoposides, Etoposide phosphates, Etoposide VP-16s, Exemestanes, Floxuridines, Fludarabines, Fluorouracils, 5-Fluorouracils, Fulvestrants, Gemcitabines, Gemtuzumabs, Ozogamicins, Gemtuzumab ozogamicins, Hydroxyureas, Idarubicins, Ifosfamides, Imatinib mesylates, Irinotecans, Letrozoles, Leucovorins, Levamisoles, Lomustines, CCNUs, Mechlorethamines, Nitrogen mustards, Megestrols, Megestrol acetates, Melphalans, L-PAMs, Mercaptopurines, 6-Mercaptopurines, Mesnas, Methotrexates, Methoxsalens, Mitomycins, Mitomycin C's, Mitotanes, Mitoxantrones, Nandrolones, Nandrolone Phenpropionates, Nofetumomabs, Oprelvekins, Oxaliplatins, Paclitaxels, Pamidronates, Pegademases, Pentostatins, Pipobromans, Plicamycins, Mithramycins, Porfimers, Porfimer sodiums, Procarbazines, Quinacrines, Rasburicases, Rituximabs, Sargramostims, Streptozocins, Talcs, Tamoxifens, Temozolomides, Teniposides, Testolactones, Thioguanines, 6-Thioguanines, Triethylenethiophosphoramides (Thiotepas), Topotecans, Toremifenes, Trastuzumabs, Tretinoins, Uracil Mustards, Valrubicins, Vinblastines, Vincristines, Vinorelbines, Zoledronates, Acivicins, Aclarubicins, Acodazoles, Acronines, Adozelesins, Aldesleukins, Retinoic Acids, Alitretinoins, 9-Cis-Retinoic Acids,

Alvocidibs, Ambazones, Ambomycins, Ametantrones, Aminoglutethimides, Amsacrines, Anaxirones, Ancitabines, Anthramycins, Apaziquones, Argimesnas, Asperlins, Atrimustines, Azacitidines, Azetepas, Azotomycins, Banoxantrones, Batabulins, Batimastats, Benaxibines, Bendamustines, Benzodepas, Bicalutamides, Bietaserpines, Biricodars, Bisantrenes, Bisnafide Dimesylates, Bizelesins, Bortezomibs, Brequinars, Bropirimines, Budotitanes, Cactinomycins, Canertinibs, Caracemides, Carbetimers, Carboquones, Carmofurs, Carubicins, Carzelesins, Cedefingols, Cemadotins, Chlorambucils, Cioteronels, Cirolemycins, Clanfenurs, Clofarabines, Crisnatols, Decitabines, Dexniguldipines, Dexormaplatins, Dezaguanines, Diaziquones, Dibrospidiums, Dienogests, Dinalins, Disermolides, Dofequidars, Doxifluridines, Droloxifenes, Duazomycins, Ecomustines, Edatrexates, Edotecarins, Eflornithines, Elacridars, Elinafides, Elsamitrucins, Emitefurs, Enloplatins, Enpromates, Enzastaurins, Epipropidines, Eptaloprosts, Erbulozoles, Esorubicins, Etanidazoles, Etoglucids, Etoprines, Exisulinds, Fadrozoles, Fazarabines, Fenretinides, Fluoxymesterones, ~~Fluorocitabines~~Flurocitabines, Fosquidones, Fostriecins, Fotretamines, Galarubicins, Galocitabines, Geroquinols, Gimatecans, Gimeracils, Gloxazones, Glufosfamides, Ilmofosines, Ilomastats, Imexons, Improsulfans, Indisulams, Inproquones, Interleukins, Interleukin-2s, recombinant Interleukins, Intoplicines, Iobenguanes, Iproplatins, Irsogladines, Ixabepilones, Ketotrexates, L-Alanosines, Lanreotides, Lapatinibs, Ledoxantrones, Leuprolides, Leuprorelins, Lexacalcitols, Liarozoles, Lobaplatins, Lometrexols, Lonafarnibs, Losoxantrones, Lurtotecans, Mafosfamides, Mannosulfans, Marimastats, Masoprocols, Maytansines, Mechlorethamines, Melengestrols, Melphalans, Menogarils, Mepitiostanes, Metesinds, Metomidates, Metoprines, Meturedepas, Miboplatins, Miproxifenes, Misonidazoles, Mitindomides, Mitocarcins, Mitocromins, Mitoflaxones, Mitogillins, Mitoguazones, Mitomalcins, Mitonafides, Mitoquidones, Mitospers, Mitozolomides, Mivobulins, Mizoribines, Mofarotenes, Mopidamols, Mubritinibs, Mycophenolic Acids, Nedaplatins, Neizarabines, Nemorubicins, Nitracrines, Nocodazoles, Nogalamycins, Nolatrexeds, Nortopixantrones, Ormaplatins, Ortataxels, Oteracils, Oxisurans, Oxophenarsines, Patupilones, Peldesines, Peliomycins, Pelitrexols, Pemetrexeds, Pentamustines, Peplomycins, Perfosfamides, Perifosines, Picoplatins, Pinafides, Piposulfans, Pirfenidones, Piroxantrones, Pixantrones, Plevitrexeds, Plomestanes, Porfiromycins, Prednimustines, Propamidines, Prospidiums, Pumitepas, Puromycins, Pyrazofurins, Ranimustines, Riboprines, Ritrosulfans, Rogletimides, Roquinimexs, Rufocromomycins, Sabarubicins, Safingols, Satraplatins, Sebriplatins, Semustines, Simtrazenes, Sizofirans, Sobuzoxanes, Sorafenibs, Sparfosates, Sparfosic Acids, Sparsomycins, Spirogermaniums, Spiromustines, Spiroplatins, Squalamines, Streptonigrins, Streptovarycins, Sufosfamides, Sulofenurs, Tacedinalines, Talisomycins, Tallimustines, Tariquidars, Tauromustines, Tecogalans, Tegafurs, Teloxantrones, Temoporfins, Teroxirones, Thiamiprines, Tiamiprines, Tiazofurins, Tilomisoles, Tilorones, Timcodars, Timonacics, Tirapazamines, Topixantrones, Trabectedins, Ecteinascidin 743, Trestolones, Triciribines, Trilostanes, Trimetrexates, Triplatin Tetranitrates, Triptorelins, Trofosfamides, Tubulozoles, Ubenimexs, Uredepas, Valspodars, Vapreotides, Verteporfins, Vinblastines, Vindesines, Vinepidines, Vinflunines, Vinformides, Vinglycinates, Vinleucinols, Vinleurosines, Vinrosidines, Vintriptols, Vinzolidines, Vorozoles, Xanthomycin A's, Guamecyclines, Zeniplatins, Zilascorbs [2-H], Zinostatins, Zorubicins, Zosuquidars, Acetazolamides, Acyclovirs, Adipiodones, Alatrofloxacins, Alfentanils, Allergenic extracts, Alpha 1-proteinase inhibitors, Alprostadils, Amikacins, Amino acids, Aminocaproic acids, Aminophyllines, Amitriptylines, Amobarbitals, Amrinones, Analgesics, Anti-poliomyelitic vaccines, Anti-rabic serums, Anti-tetanus immunoglobulins, tetanus vaccines, Antithrombin IIIs, Antivenom serums, Argatrobans,

Arginines, Ascorbic acids, Atenolols, Atracuriums, Atropines, Aurothioglucoses, Azathioprines, Aztreonams, Bacitracins, Baclofens, Basiliximabs, Benzoic acids, Benztropines, Betamethasones, Biotins, Bivalirudins, Botulism antitoxins, Bretyliums, Bumetanides, Bupivacaines, Buprenorphines, Butorphanols, Calcitonins, Calcitriols, Calciums, Capreomycins, Carboprosts, Carnitines, Cefamandoles, Cefoperazones, Cefotaximes, Cefoxitins, Ceftizoximes, Cefuroximes, Chloramphenicols, Chloroprocaines, Chloroquines, Chlorothiazides, Chlorpromazines, Chondroitinsulfuric acids, Choriogonadotropin alfas, Chromiums, Cidofovirs, Cimetidines, Ciprofloxacins, Cisatracuriums, Clonidines, Codeines, Colchicines, Colistins, Collagens, Corticorelin ovine triflutates, Corticotrophins, Cosyntropins, Cyanocobalamins, Cyclosporines, Cysteines, Dacliximabs, Dalfopristins, Dalteparins, Danaparoids, Dantrolenes, Deferoxamines, Desmopressins, Dexamethasones, Dexmedetomidines, Dexpanthenols, Dextrans, Iron dextrans, Diatrizoic acids, Diazepams, Diazoxides, Dicyclomines, Digibinds, Digoxins, Dihydroergotamines, Diltiazems, Diphenhydramines, Dipyridamoles, Dobutamines, Dopamines, Doxacuriums, Doxaprams, Doxercalciferols, Doxycyclines, Droperidols, Dyphyllines, Edetic acids, Edrophoniums, Enalaprilats, Ephedrines, Epoprostenols, Ergocalciferols, Ergonovines, Ertapenems, Erythromycins, Esmolols, Estradiols, Estrogenics, Ethacrynic acids, Ethanolamines, Ethanols, Ethiodized oils, Etidronic acids, Etomidates, Famotidines, Fenoldopams, Fentanyls, Flumazenils, Fluoresceins, Fluphenazines, Folic acids, Fomepizoles, Fomivirsens, Fondaparinuxs, Foscarnets, ~~Fosphenyloins~~Fosphenytoins, Furosemides, Gadoteridols, Gadoversetamides, Ganciclovirs, Gentamicins, Glucagons, Glucoses, Glycines, Glycopyrrolates, Gonadorelins, Gonadotropin chorionics, Haemophilus B polysaccharides, Hemins, Herbals, Histamines, Hydralazines, Hydrocortisones, Hydromorphones, Hydroxocobalamins, Hydroxyzines, Hyoscyamines, Ibutilides, Imiglucerases, Indigo carmines, Indomethacins, Iodides, Iopromides, Iothalamic acids, Ioxaglic acids, Ioxilans, Isoniazids, Isoproterenols, Japanese encephalitis vaccines, Kanamycins, Ketamines, Labetalols, Lepirudins, Levobupivacaines, Levothyroxines, Lincomycins, Liothyronines, Luteinizing hormones, Lyme disease vaccines, Mangafodipirs, Manthtols, Meningococcal polysaccharide vaccines, Meperidines, Mepivacaines, Mesoridazines, Metaraminols, Methadones, Methocarbamols, Methohexitals, Methyldopates, Methylergonovines, Metoclopramides, Metoprolols, Metronidazoles, Minocyclines, Mivacuriums, Morrhuic acids, Moxifloxacins, Muromonab-CD3s, Mycophenolate mofetils, Nafcillins, Nalbuphines, Nalmefenes, Naloxones, Neostigmines, Niacinamides, Nicardipines, Nitroglycerins, Nitroprussides, Norepinephrines, Orphenadrines, Oxacillins, Oxymorphones, Oxytetracyclines, Oxytocins, Pancuroniums, Panthenols, Pantothenic acids, Papaverines, Peginterferon alpha 2As, Penicillin Gs, Pentamidines, Pentazocines, Pentobarbitals, Perflutrens, Perphenazines, Phenobarbitals, Phentolamines, Phenylephrines, ~~Phenyloins~~Phenytoins, Physostigmines, Phytonadiones, Polymyxin, Pralidoximes, ~~Prilocalnes~~Prilocaines, Procainamides, Procaines, Prochlorperazines, Progesterones, Propranolols, Pyridostigmine hydroxides, Pyridoxines, Quinidines, Quinupristins, Rabies immunoglobulins, Rabies vaccines, Ranitidines, Remifentanils, Riboflavins, Rifampins, Ropivacaines, Samariums, Scopolamines, Seleniums, Sermorelins, Sincalides, Somatrems, Spectinomycins, Streptokinases, Streptomycins, Succinylcholines, Sufentanils, Sulfamethoxazoles, Tacrolimuses, Terbutalines, Teriparatides, Testosterones, Tetanus antitoxins, Tetracaines, Tetradecyl sulfates, Theophyllines, Thiamines, Thiethylperazines, Thiopentals, Thyroid stimulating hormones, Tinzaparins, Tirofibans, Tobramycins, Tolazolines, Tolbutamides, Torsemides, Tranexamic acids, Treprostinils, Trifluoperazines, Trimethobenzamides, Trimethoprims, Tromethamines, Tuberculins, Typhoid vaccines,

Urofollitropins, Urokinases, Valproic acids, Vasopressins, Vecuroniums, Verapamils, Voriconazoles, Warfarins, Yellow fever vaccines, Zidovudines, Zincs, Ziprasidone hydrochlorides, Aclacinomycins, Actinomycins, Adriamycins, Azaserines, 6-Azauridines, Carzinophilins, Chromomycins, Denopterins, 6- Diazo 5 Oxo-L-Norleucines, Enocitabines, Floxuridines, Olivomycins, Pirarubicins, Piritreximes, Pteropterins, Tegafurs, Tubercidins, Alteplases, Arcitumomabs, bevacizumabs, Botulinum Toxin Type A's, Botulinum Toxin Type B's, Capromab Pendetides, Daclizumabs, Dornase alphas, Drotrecogin alphas, Imciromab Pentetates, Iodine-131's, an antibiotic agent; an angiogenesis inhibitor; anti-cataract and anti-diabetic retinopathy substances; carbonic anhydrase inhibitors; mydriatics; photodynamic therapy agents; prostaglandin analogs; growth factor; anti-neoplastics; anti-metabolites; anti-viral; amebicides and anti-protozoals; anti-tuberculosis and anti-leprotic; antitoxins and antivenins; antihemophilic factor, anti-inhibitor coagulant complex, antithrombin III, coagulations Factor V, coagulation Factor IX, plasma protein fraction, von Willebrand factor; antiplatelet agent a colony stimulating factor (CSF); an erythropoiesis stimulator; hemostatics and albumins; Immune Globulins; thrombin inhibitors; anticoagulants; a steroidal anti-inflammatory drug selected from among alclometasones, algestones, beclomethasones, betamethasones, budesonides, clobetasols, clobetasones, clocortolones, cloprednols, corticosterones, cortisones, cortivazols, deflazacorts, desonides, desoximetasones, dexamethasones, diflorasones, diflucortolones, difluprednates, enoxolones, fluazacorts, flucloronides, flumethasones, flunisolides, fluocinolones, fluocinonides, fluocortins, fluocortolones, fluorometholones, fluperolones, fluprednidenes, fluprednisolones, flurandrenolides, fluticasones, formocortals, halcinonides, halobetasols, halometasones, halopredones, hydrocortamates, hydrocortisones, loteprednol etabonate, mazipredones, medrysones, meprednisones, methylprednisolones, mometasone furoate, paramethasones, prednicarbates, prednisolones, prednisones, prednivals, prednylidenes, rimexolones, tixocortols and triamcinolones; Docosanols, prostaglandins, prostaglandin analogs, antiprostaglandins and prostaglandin precursors; miotics, cholinergics and anti-cholinesterase; and anti-allergenics.

~~[0042]~~(35) The compositions and modified PH20 polypeptides can be used to treat any condition normally treated by the PH20 polypeptide or the therapeutically active agent. These include, for example, conditions in which hyaluronan plays a role or is associated with the etiology of the disease due to, for example, accumulation or overproduction of hyaluronan. Hence provided are methods, uses of the compositions and modified PH20 polypeptides for treating a hyaluronan-associated disease or condition by administering any of the modified PH20 polypeptides or compositions provided herein. Hyaluronan-associated diseases and conditions include, for example, inflammatory disease and tumors or cancers, including a late-stage cancer, metastatic cancers and undifferentiated cancers, such as ovarian cancer, in situ carcinoma (ISC), squamous cell carcinoma (SCC), prostate cancer, pancreatic cancer, non-small cell lung cancer, breast cancer and colon cancer. The PH20 polypeptide can be modified to exhibit increased half-life for such treatments. For example, the PH20 polypeptide can be modified with a polymer such as a PEG moiety for such treatments.

~~[0043]~~(36) Also provided are methods for increasing delivery of a therapeutic agent to a subject by: administering to a subject any of the modified PH20 polypeptides or compositions provided herein, and administering the therapeutic agent. The therapeutic agent can be administered in the same composition or separately, and can be administered before or after, simultaneously, or intermittently, with administration of the PH20 polypeptide(s). Administration includes any

route, including intravenous and subcutaneous administration, such as simultaneously with, intermittently with, or subsequent to administration of the therapeutic agent. The therapeutic agents include any of those set forth above, elsewhere herein and/or known to those of skill in the art.

~~[0044]~~(37) Also provided are methods for treating an excess of glycosaminoglycans; for treating a tumor; for treating glycosaminoglycan accumulation in the brain; for treating a cardiovascular disorder; for treating an ophthalmic disorder; for treating pulmonary disease; for increasing penetration of chemotherapeutic agents into solid tumors; for treating cellulite; for treating a proliferative disorder; or for increasing bioavailability of drugs and other therapeutic agents by administering the modified PH20 polypeptides or compositions provided herein.

~~[0045]~~(38) Also provided are pharmaceutical compositions for use in treating a hyaluronan-associated disease or disorder; for use in delivering a therapeutic agent to a subject; for treating an excess of glycosaminoglycans; for treating a tumor; for treating glycosaminoglycan accumulation in the brain; for treating a cardiovascular disorder; for treating an ophthalmic disorder; for treating pulmonary disease; for increasing penetration of chemotherapeutic agents into solid tumors; for treating cellulite; for treating a proliferative disorder; or for increasing bioavailability of drugs and other therapeutic agents; and for any other use of compositions containing PH20 polypeptides.

~~[0046]~~(39) Provided herein is a method for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability under a denaturation condition that includes the steps of: a) testing the activity of a modified hyaluronan-degrading enzyme in a composition containing a denaturing agent and/or under a denaturing condition; b) testing the activity of the modified hyaluronan-degrading enzyme in the same composition and/or under the same conditions as a) except absent the denaturing agent or condition; and c) selecting or identifying a modified hyaluronan-degrading enzyme that exhibits activity in a) that is at least 5% of the activity in b). In such an example, the activity is hyaluronidase activity. In some examples of the methods, a modified hyaluronan-degrading enzyme is selected or identified if the activity in a) is at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the activity in b), for example, a modified hyaluronan-degrading enzyme is selected or identified if the activity in a) is at least 40% or more of the activity in b). The method also can include steps of: d) comparing the activity of the modified ~~hyaluronan-degrading~~hyaluronan-de grading enzyme in a) to the activity of the unmodified hyaluronan-degrading enzyme tested under the same conditions; and e) identifying or selecting a modified hyaluronan-degrading enzyme that exhibits at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the hyaluronidase activity compared to the unmodified hyaluronan-degrading enzyme.

~~[0047]~~(40) Also provide herein is a method for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability, such as increased stability, under a denaturation condition, that includes the steps of: a) testing the activity of a modified hyaluronan-degrading enzyme in a composition containing a denaturing agent and/or under a denaturing condition; b) testing the activity of the corresponding unmodified hyaluronan-degrading enzyme in a composition containing the same denaturing agent and/or under the same denaturing condition as

a), whereby the activity is tested under the same conditions as a); and c) selecting or identifying a modified hyaluronan-degrading enzyme that exhibits greater activity than the unmodified hyaluronan-degrading enzyme, thereby identifying or selecting a modified hyaluronan-degrading enzyme that exhibits increased stability under a denaturation condition. In such an example, the activity can be a hyaluronidase activity. In examples of the method, a modified hyaluronan-degrading enzyme is selected or identified if the activity is at least 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% or more of the activity compared to the unmodified hyaluronan-degrading enzyme. In such an example, the method also can include additional steps of: d) testing the activity of the selected or identified modified hyaluronan-degrading enzyme in a composition containing a denaturing agent and/or under a denaturing condition; e) testing the activity of the same selected or identified modified hyaluronan-degrading enzyme in the same composition and/or under the same conditions as d) except absent the denaturing agent or condition; and f) selecting or identifying a modified hyaluronan-degrading enzyme that exhibits activity in d) that is at least 5% of the activity in e). In such an example, the activity is hyaluronidase activity. In some examples of the methods, a modified hyaluronan-degrading enzyme is selected or identified if the activity in d) is at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the activity in e), for example, a modified hyaluronan-degrading enzyme is selected or identified if the activity in d) is at least 40% or more of the activity in e).

~~[0048]~~(41) In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, the denaturing agent or condition is caused by temperature, agitation, no or low salt or the presence of an excipient. For example, the denaturing agent or condition is caused by elevated temperature that is from or from about 30~~.degree.~~° C. to 42~~.degree.~~° C., such as greater than or greater than about 30~~.degree.~~° C., 31~~.degree.~~° C., 32~~.degree.~~° C., 33~~.degree.~~° C., 34~~.degree.~~° C., 35~~.degree.~~° C., 36~~.degree.~~° C., 37~~.degree.~~° C., 38~~.degree.~~° C., 39~~.degree.~~° C., 40~~.degree.~~° C., 41~~.degree.~~° C. or 42~~.degree.~~° C. In other examples, the denaturing agent or condition is the absence of salt or low salt less than 100 mM, such as low salt less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM. In further examples, the denaturing agent or condition is a denaturing excipient selected from among an antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents and sweeteners.

~~[0049]~~(42) In particular examples of any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, the denaturing agent or condition is a preservative(s), for example, a phenolic preservative(s). The phenolic preservative(s) can be a phenol, metacresol (m-cresol), benzyl alcohol, or a paraben. For example, the denaturing agent or condition is a preservative(s) that is phenol and/or m-cresol. In such examples, the total amount of phenolic preservative in the composition, as a percentage (%) of mass concentration (w/v), is from or from about 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive.

~~[0050]~~(43) In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, prior to testing the activity of a hyaluronan-degrading enzyme composition in a) and/or b), the hyaluronan-degrading enzyme is exposed to the denaturation condition or denaturing agent for a predetermined time. The predetermined time is a time period

that is user selected depending on the particular ~~hyaluronan-degrading~~hyaluronan-de grading enzyme that is being evolved or selected, the particular denaturation condition or denaturing agent, the amount or extent of the denaturation condition or denaturing agent, the application or use of the hyaluronan-degrading enzyme and other similar factors. For example, the predetermined time can be from or from about 1 minute to 1 month, 1 minute to 3 weeks, 1 minute to 2 weeks, 1 minute to 1 week, 1 minute to 24 hours, 1 minute to 12 hours, 30 minutes to 6 hours or 1 hour to 4 hours, such as at least or about at least 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, two days, three days, four days, five days, six days, 7 days, two weeks or one month.

~~[0051]~~(44) In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme, the modified hyaluronan-degrading enzyme is one that contains an amino acid replacement, insertion or deletion of amino acids compared to an unmodified hyaluronan-degrading enzyme. For example, the modified hyaluronan-degrading enzyme contains an amino acid replacement, such as a single amino acid replacement or two, three, four, five, six, seven, eight, nine or more amino acid replacements compared to an unmodified form of the hyaluronan-degrading enzyme. In particular aspects of the method, a library or collection of modified hyaluronan-degrading enzymes are screened in order to evolve or identify or select a modified hyaluronan-degrading enzyme that exhibits stability, such as increased stability, under a denaturation condition. Thus, in examples of the methods herein, a plurality of modified hyaluronan-degrading enzymes are tested in a) and/or b). In such examples, the plurality of modified hyaluronan-degrading enzymes are modified compared to the corresponding unmodified hyaluronan-degrading enzyme to generate a collection of modified hyaluronan-degrading enzymes, whereby each modified protein in the collection is tested in each of a) and/or b). In the collection or library, each modified hyaluronan-degrading enzyme contains a single amino acid replacement compared to the unmodified form of the hyaluronan-degrading enzyme, such that the plurality of modified enzymes are such that the amino acid at each modified position is replaced by up to 1-19 other amino acids other than the original amino acid at the position, whereby each modified hyaluronan-degrading enzyme contains a different amino acid replacement, and every amino acid along the length of the hyaluronan-degrading enzyme, or a selected portion thereof, is replaced.

~~[0052]~~(45) In any of the methods provided herein, the modified hyaluronan-degrading enzyme is modified compared to an unmodified ~~hyaluronan-degrading~~hyaluronan-de grading enzyme by insertion, deletion or replacement of an amino acid(s). The unmodified hyaluronan-degrading enzyme can be a chondroitinase or can be a hyaluronidase. In examples herein, the unmodified hyaluronidase is a PH20 hyaluronidase or truncated form thereof lacking a C-terminal glycosylphosphatidylinositol (GPI) anchor attachment site or a portion of the GPI anchor attachment site, whereby the truncated form exhibits hyaluronidase activity. PH20 hyaluronidase can be a human, monkey, bovine, ovine, rat, fox, mouse or guinea pig PH20. In particular examples, the PH20 hyaluronidase is a human PH20 or a C-terminal truncated form thereof. For example, the unmodified hyaluronan-degrading enzyme is one that has the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, 870 or a sequence of amino acids that is at least 80% sequence identity to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, 870, such as at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to any of SEQ ID NOS: 3, 7, 10, 12, 14, 24, 32-66, 69, 72, 857, 859, 861, or 870. In particular examples, the unmodified

hyaluronan-degrading enzyme is a PH20 hyaluronidase having the sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a sequence of amino acids that exhibits at least 85% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, such as a sequence of amino acids that exhibits at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72.

~~[0053]~~(46) In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability, the method is performed in vitro. Also provided are any of the methods that are iterative, whereby the steps of the method are repeated a plurality of times, wherein in each repetition, further modified hyaluronan-degrading enzymes of a selected modified hyaluronan-degrading enzyme are generated and tested, whereby the modified hyaluronan-degrading enzyme is evolved to exhibit increased stability under a denaturation condition. Also provided herein is a modified hyaluronan-degrading enzyme identified by any of the methods provided herein.

Description

BRIEF DESCRIPTION OF THE FIGURES

~~[0054]~~(1) FIG. 1 depicts the amino acid sequence of full-length human PH20 (set forth in SEQ ID NO:7) and soluble C-terminal truncated variants thereof. The C-terminal amino acid residue of exemplary C-terminal truncated variants of full-length PH20 are indicated by bold font. The complete amino acid sequences of exemplary C-terminal truncated variants of full-length PH20 also are provided in SEQ ID NOS: 3 and 32-66. The C-terminal amino acid residue of an exemplary soluble PH20, whose complete sequence is set forth in SEQ ID NO:3, also is indicated by underline. Exemplary, non-limiting, positions for amino acid replacements are indicated by highlighting. Corresponding positions can be identified by alignment of a sequence of interest with any of SEQ ID NOS: 3, 7 or 32-66, and in particular with SEQ ID NO:3.

~~[0055]~~(2) FIG. 2 (A-L) depicts exemplary alignments of human soluble PH20 set forth in SEQ ID NO:3 with other PH20 polypeptides. A "*" means that the aligned residues are identical, a ":" means that aligned residues are not identical, but are similar and contain conservative amino acids residues at the aligned position, and a "." means that the aligned residues are similar and contain semi-conservative amino acid residues at the aligned position. Exemplary, non-limiting, corresponding positions for amino acid replacements are indicated by highlighting. For example, FIG. 2A depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with chimpanzee PH20 set forth in SEQ ID NO:10. FIG. 2B depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Rhesus monkey PH20 set forth in SEQ ID NO:12. FIG. 2C depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Cynomolgus monkey PH20 set forth in SEQ ID NO:14. FIG. 2D depicts the alignment of human soluble PH20 set forth in SEQ ID NO:3 with bovine PH20 set forth in SEQ ID NO:16. FIG. 2E depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with mouse PH20 set forth in SEQ ID NO:20. FIG. 2F depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with rat PH20 set forth in SEQ ID NO:22. FIG. 2G depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with rabbit PH20 set forth in SEQ ID NO:24. FIG. 2H

depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with guinea pig PH20 set forth in SEQ ID NO:29. FIG. 2I depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Fox PH20 set forth in SEQ ID NO:31. FIG. 2J depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Gibbon PH20 set forth in SEQ ID NO:857. FIG. 2K depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Marmoset PH20 set forth in SEQ ID NO:859. FIG. 2L depicts the alignment of a human soluble PH20 set forth in SEQ ID NO:3 with Orangutan PH20 set forth in SEQ ID NO:861.

DETAILED DESCRIPTION

Outline

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A. DefinitionsDEFINITIONS

[0112](4) Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

[0113](5) As used herein, a hyaluronan-degrading enzyme refers to an enzyme that catalyzes the cleavage of a hyaluronan polymer (also referred to as hyaluronic acid or HA) into smaller molecular weight fragments. Exemplary hyaluronan-degrading enzymes are hyaluronidases, and particular chondroitinases and lyases that have the ability to depolymerize hyaluronan. Exemplary chondroitinases that are hyaluronan-degrading enzymes include, but are not limited to, chondroitin ABC lyase (also known as chondroitinase ABC), chondroitin AC lyase (also known as chondroitin sulfate lyase or chondroitin sulfate eliminase) and chondroitin C lyase. Chondroitin ABC lyase contains two enzymes, chondroitin-sulfate-ABC endolyase (EC 4.2.2.20) and chondroitin-sulfate-ABC exolyase (EC 4.2.2.21). An exemplary chondroitin-sulfate-ABC endolyases and chondroitin-sulfate-ABC exolyases include, but are not limited to, those from Proteus vulgaris and Pedobacter heparinus (the Proteus vulgaris chondroitin-sulfate-ABC endolyase is set forth in SEQ ID NO:922; Sato et al. (1994) Appl. Microbiol. Biotechnol. 41(1):39-46). Exemplary chondroitinase AC enzymes from bacteria include, but are not limited to, those from Pedobacter heparinus, set forthfor th in SEQ ID NO: 923, Victivallis vadensis, set forth in SEQ ID NO:924, and Arthrobacter aurescens (Tkalec et al. (2000) Applied and Environmental Microbiology 66(1):29-35; Ernst et al. (1995) Critical Reviews in Biochemistry and Molecular Biology 30(5):387-444). Exemplary chondroitinase C enzymes from bacteria include, but are not limited to, those from Streptococcus and Flavobacterium (Hibi et al. (1989) FEMS-Microbiol-Lett. 48(2):121-4; Michelacci et al. (1976) J. Biol. Chem. 251:1154-8; Tsuda et al. (1999) Eur. J. Biochem. 262:127-133).

[0114](6) As used herein, hyaluronidase refers to a class of enzymes that degrade hyaluronan. Hyaluronidases include, but are not limited to, bacterial hyaluronidases (EC 4.2.2.1 or EC 4.2.99.1), hyaluronidases from leeches, other parasites and crustaceans (EC 3.2.1.36), and mammalian-type hyaluronidases (EC 3.2.1.35). Hyaluronidases include any of non-human origin including, but not limited to, murine, canine, feline, leporine, avian, bovine, ovine, porcine, equine, piscine, ranine, bacterial, and any from leeches, other parasites, and crustaceans. Exemplary human hyaluronidases include HYAL1, HYAL2, HYAL3, HYAL4, and PH20. Also included amongst hyaluronidases are soluble hyaluronidases, including, ovine and bovine PH20, and soluble PH20. Exemplary hyaluronidases include any set forth in SEQ ID NOS: 6, 7-31, 69, 70, 71, 72, 856-861, 869-921, mature forms thereof (lacking the signal sequence), or allelic or species variants thereof. Hyaluronidases also include truncated forms thereof that exhibit hyaluronidase activity, including C-terminal truncated variants that are soluble.

[0115](7) As used herein, PH20 refers to a type of hyaluronidase that occurs in sperm and is neutral-active. PH-20 occurs on the sperm surface, and in the lysosome-derived acrosome, where

it is bound to the inner acrosomal membrane. PH20 includes those of any origin including, but not limited to, human, chimpanzee, Cynomolgus monkey, Rhesus monkey, murine, bovine, ovine, guinea pig, rabbit and rat origin. Exemplary PH20 polypeptides, including precursor and mature forms, include those from human (SEQ ID NO:6 and 7), chimpanzee (SEQ ID NO:8, 9, 10, 869 and 870), Rhesus monkey (SEQ ID NO:11 and 12), Cynomolgus monkey (SEQ ID NO:13 and 14), cow (e.g., SEQ ID NOS:15-18); mouse (SEQ ID NO:19 and 20); rat (SEQ ID NO:21 and 22); rabbit (SEQ ID NO:23 and 24); sheep (SEQ ID NOS:25-27), guinea pig (SEQ ID NO:28 and 29); fox (SEQ ID NO: 30 and 31); Gibbon (SEQ ID NO:856 and 857), Marmoset (SEQ ID NO:858 and 859) and orangutan (SEQ ID NO:860 and 861). Reference to PH20 includes precursor PH20 polypeptides and mature PH20 polypeptides (such as those in which a signal sequence has been removed), truncated forms thereof that have activity, and includes allelic variants and species variants, variants encoded by splice variants, and other variants, including polypeptides that have at least 40%, 45%, 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the precursor polypeptides set forth in SEQ ID NO:7, or the mature forms thereof. PH20 polypeptides also include those that contain chemical or posttranslational modifications and those that do not contain chemical or posttranslational modifications. Such modifications include, but are not limited to, PEGylation, albumination, glycosylation, farnysylation, carboxylation, hydroxylation, phosphorylation, and other polypeptide modifications known in the art. Examples of commercially available bovine or ovine soluble hyaluronidases are Vitrase~~.RTM.~~® hyaluronidase (ovine hyaluronidase) and Amphadase~~.RTM.~~® hyaluronidase (bovine hyaluronidase).

~~[0116]~~(8) As used herein, a soluble PH20 refers to a polypeptide characterized by its solubility under physiological conditions. Generally, a soluble PH20 lacks all or a portion of a glycophosphatidyl anchor (GPI) attachment sequence, or does not otherwise sufficiently anchor to the cell membrane. For example, a soluble PH20 can be a C-terminally truncated variant of a PH20 lacking a contiguous sequence of amino acids that corresponds to all or a portion of a glycophosphatidyl anchor (GPI) attachment sequence. Hence, upon expression from a cell, a soluble PH20 is secreted into the medium. Soluble PH20 proteins can be distinguished, for example, by its partitioning into the aqueous phase of a Triton X-114 solution warmed to 37~~.degree.~~° C. (Bordier et al., (1981) J. Biol. Chem., 256:1604-7). Membrane-anchored, such as lipid anchored hyaluronidases, will partition into the detergent rich phase, but will partition into the detergent-poor or aqueous phase following treatment with Phospholipase-C. Included among soluble PH20 hyaluronidases are membrane anchored hyaluronidases in which one or more regions associated with anchoring of the hyaluronidase to the membrane has been removed or modified, where the soluble form retains hyaluronidase activity. Soluble hyaluronidases include recombinant soluble hyaluronidases and those contained in or purified from natural sources, such as, for example, testes extracts from sheep or cows. Exemplary of such soluble hyaluronidases are soluble human PH20 (SEQ ID NO: 3 or 32-66). Other soluble hyaluronidases include ovine (SEQ ID NO:25-27) and bovine (SEQ ID NO:16 or 18) PH20.

~~[0117]~~(9) As used herein, soluble human PH20 (sHuPH20) includes human PH20 polypeptides that lack a contiguous sequence of amino acids from the C-terminus of human PH20 that includes all or a portion of the glycosylphosphatidylinositol (GPI) anchor sequence (C-terminally truncated PH20 polypeptides) such that upon expression, the polypeptides are soluble under physiological conditions. For example, soluble human PH20 polypeptides are C-terminally truncated polypeptides of human PH20 set forth as SEQ ID NO:6 in its precursor form or in SEQ

ID NO:7 in its mature form lacking the signal sequence, or allelic variants thereof (e.g. set forth in any of SEQ ID NOS: 68-72). Solubility can be assessed by any suitable method that demonstrates solubility under physiologic conditions. Exemplary of such methods is the Triton~~.RTM.~~® X-114 assay, that assesses partitioning into the aqueous phase and that is described above. In addition, a soluble human PH20 polypeptide is, if produced in CHO cells, such as ~~CHO-S~~CHO—S cells, a polypeptide that is expressed and is secreted into the cell culture medium. Soluble human PH20 polypeptides, however, are not limited to those produced in CHO cells, but can be produced in any cell or by any method, including recombinant expression and polypeptide synthesis. Reference to secretion in CHO cells is definitional. Hence, if a polypeptide could be expressed and secreted in CHO cells and is soluble in the media, i.e., partitions into the aqueous phase when extracted with Triton~~.RTM.~~® X-114, it is a soluble PH20 polypeptide whether or not it is so-produced. The precursor polypeptides for sHuPH20 polypeptides can include a signal sequence, such as a heterologous or non-heterologous (i.e., native) signal sequence. Exemplary of the precursors are those that include a signal sequence, such as the native 35 amino acid signal sequence at amino acid positions 1-35 (see, e.g., amino acids 1-35 of SEQ ID NO:6).

~~[0118]~~(10) As used herein, ~~"~~"native~~"~~" or ~~"~~"wildtype~~"~~" with reference to a PH20 polypeptide refers to a PH20 polypeptide encoded by a native or naturally occurring PH20 gene, including allelic variants, that is present in an organism, including a human and other animals, in nature. Reference to wild-type PH20 without reference to a species is intended to encompass any species of a wild-type PH20. Included among wild-type PH20 polypeptides are the encoded precursor polypeptide, fragments thereof, and processed forms thereof, such as a mature form lacking the signal peptide as well as any pre- or post-translationally processed or modified forms thereof. Also included among native PH20 polypeptides are those that are post-translationally modified, including, but not limited to, those that are modified by glycosylation, carboxylation and/or hydroxylation. The amino acid sequences of exemplary wild-type human PH20 are set forth in SEQ ID NOS: 6 and 7 and those of allelic variants, including mature forms thereof, are set forth in SEQ ID NOS:68-72. Other animals produce native PH20, including, but not limited to, native or wildtype sequences set forth in any of SEQ ID NOS: 8-31, 856-861, 869 or 870.

~~[0119]~~(11) As used herein, modification is in reference to modification of a sequence of amino acids of a polypeptide or a sequence of nucleotides in a nucleic acid molecule and includes deletions, insertions, and replacements of amino acids and nucleotides, respectively. Modifications also can include post-translational modifications or other changes to the molecule that can occur due to conjugation or linkage, directly or indirectly, to another moiety. Methods of modifying a polypeptide are routine to those of skill in the art, such as by using recombinant DNA methodologies.

~~[0120]~~(12) As used herein, a ~~"~~"modified hyaluronan-degrading enzyme~~"~~" refers to a hyaluronan-degrading enzyme that contains a modification compared to a reference or unmodified hyaluronan-degrading enzyme. The modification can be an amino acid replacement (substitution), insertion (addition) or deletion of one or more amino acid residues. The amino acid residue can be a natural or non-natural amino acid. In some cases, the modification can be a post-translational modification. A modified hyaluronan-degrading enzyme can have up to 150 amino acid differences compared to a reference or unmodified hyaluronan-degrading enzyme, so long as the resulting modified hyaluronan-degrading enzyme exhibits hyaluronidase activity.

Typically, a modified hyaluronan-degrading enzyme contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acid modifications.

~~[0121]~~(13) As used herein, an unmodified hyaluronan-degrading enzyme refers to a starting polypeptide that is selected for modification as provided herein. The starting polypeptide can be a naturally-occurring, wild-type form of a polypeptide. In addition, the starting polypeptide can be altered or mutated, such that it differs from a native wild type isoform but is nonetheless referred to herein as a starting unmodified polypeptide relative to the subsequently modified polypeptides produced herein. Thus, existing proteins known in the art that have been modified to have a desired increase or decrease in a particular activity or property compared to an unmodified reference protein can be selected and used as the starting unmodified polypeptide. For example, a protein that has been modified from its native form by one or more single amino acid changes and possesses either an increase or decrease in a desired property, such as a change in an amino acid residue or residues to alter glycosylation, can be selected for modification, and hence referred to herein as unmodified, for further modification. An unmodified hyaluronan-degrading enzyme includes human and non-human hyaluronan-degrading enzymes, including hyaluronan-degrading enzymes from non-human mammals and bacteria. Exemplary unmodified hyaluronan-degrading enzyme are any set forth in SEQ ID NOS: 2, 3, 6, 7-66, 68-72, 856-861, 869-924 or mature, C-terminally truncated forms thereof that exhibit hyaluronidase activity, or a hyaluronan-degrading enzyme that exhibits at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 2, 3, 6, 7-66, 68-72, 856-861, 869-924. It is understood that an unmodified hyaluronan-degrading enzyme generally is one that does not contain the modification(s), such as amino acid replacement(s) of a modified hyaluronan-degrading enzyme.

~~[0122]~~(14) As used herein, ~~"~~"modified PH20 polypeptide~~"~~" or ~~"~~"variant PH20 polypeptide~~"~~" refers to a PH20 polypeptide that contains at least one amino acid modification, such as at least one amino acid replacement as described herein, in its sequence of amino acids compared to a reference unmodified PH20 polypeptide. A modified PH20 polypeptide can have up to 150 amino acid replacements, so long as the resulting modified PH20 polypeptide exhibits hyaluronidase activity. Typically, a modified PH20 polypeptide contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acid replacements. It is understood that a modified PH20 polypeptide also can include any one or more other modifications, in addition to at least one amino acid replacement as described herein.

~~[0123]~~(15) As used herein, an unmodified PH20 polypeptide refers to a starting PH20 polypeptide that is selected for modification as provided herein. The starting polypeptide can be a naturally-occurring, wild-type form of a polypeptide. In addition, the starting polypeptide can be altered or mutated, such that it differs from a native wild type isoform but is nonetheless referred to herein as a starting unmodified polypeptide relative to the subsequently modified polypeptides produced herein. Thus, existing proteins known in the art that have been modified to have a desired increase or decrease in a particular activity or property compared to an unmodified reference protein can be selected and used as the starting unmodified polypeptide. For example, a protein that has been modified from its native form by one or more single amino acid changes and possesses either an increase or decrease in a desired property, such as a change

in an amino acid residue or residues to alter glycosylation, can be selected for modification, and hence referred to herein as unmodified, for further modification. Exemplary unmodified PH20 polypeptides is a human PH20 polypeptide or allelic or species variants thereof or other variants, including mature and precursor polypeptides. For example, exemplary reference PH20 polypeptides is a mature full length PH20 polypeptide set forth in SEQ ID NOS: 7, 69 or 72, or in C-terminally truncated forms thereof such as set forth in any of SEQ ID NOS: 3 and 32-66, or in a PH20 polypeptide that exhibits at least 68%, 69%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. A reference PH20 polypeptide also can include the corresponding precursor form such as set forth in any of SEQ ID NOS: 2, 6, 68, 70, 71 or other precursor forms, or in a PH20 polypeptide that exhibits at least 68%, 69%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of SEQ ID NOS: 2, 6, 68, 70, 71. It is understood that an unmodified hyaluronan-degrading enzyme generally is one that does not contain the modification(s), such as amino acid replacement(s) of a modified hyaluronan-degrading enzyme.

~~[0124]~~(16) As used herein, an N-linked moiety refers to an asparagine (N) amino acid residue of a polypeptide that is capable of being glycosylated by post-translational modification of a polypeptide. Exemplary N-linked moieties of human PH20 include amino acids N47, N131, N200, N219, N333, N358 and N365 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 (corresponding to amino acid residues N82, N166, N235, N254, N368, N393 and N490 of human PH20 set forth in SEQ ID NO: 6).

~~[0125]~~(17) As used herein, an N-glycosylated polypeptide refers to a PH20 polypeptide containing oligosaccharide linkage of at least three N-linked amino acid residues, for example, N-linked moieties corresponding to amino acid residues N200, N333 and N358 of SEQ ID NO:3 or 7. An N-glycosylated polypeptide can include a polypeptide where three, four, five and up to all of the N-linked moieties are linked to an oligosaccharide. The N-linked oligosaccharides can include oligomannose, complex, hybrid or sulfated oligosaccharides, or other oligosaccharides and monosaccharides.

~~[0126]~~(18) As used herein, an N-partially glycosylated polypeptide refers to a polypeptide that minimally contains an N-acetylglucosamine glycan linked to at least three N-linked moieties. A partially glycosylated polypeptide can include various glycan forms, including monosaccharides, oligosaccharides, and branched sugar forms, including those formed by treatment of a polypeptide with EndoH, EndoF1, EndoF2 and/or EndoF3.

~~[0127]~~(19) As used herein, ~~"~~“conditions~~"~~” refers to any parameter that can influence the activity or properties of a protein or agent. For purposes herein, conditions generally refer to the presence, including amount, of excipients, carriers or other components in a formulation other than the active agent (e.g., modified PH20 hyaluronidase); temperature; time (e.g., time of storage or exposure); storage vessel; properties of storage (e.g., agitation) and/or other conditions associated with exposure or use.

~~[0128]~~(20) As used herein, ~~"~~“denaturation~~" or "~~” or “denaturing~~"~~” or grammatical variations thereof with reference to a protein refers to a biochemical change in a protein so that a property or activity of the protein is diminished or eliminated. The biochemical change can be a change in

the tertiary structure of the protein to unfold. The property or activity can be completely abolished or can be reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more.

[0129](21) As used herein, property refers to a physical or structural property, such as the three-dimensional structure, pipI, half-life, conformation and other such physical characteristics. For example, a change in a property can be manifested as the solubility, aggregation or crystallization of a protein.

[0130](22) As used herein, activity refers to a functional activity or activities of a polypeptide or portion thereof associated with a full-length (complete) protein. Functional activities include, but are not limited to, biological activity, catalytic or enzymatic activity, antigenicity (ability to bind or compete with a polypeptide for binding to an anti-polypeptide antibody), immunogenicity, ability to form multimers, and the ability to specifically bind to a receptor or ligand for the polypeptide.

[0131](23) As used herein, hyaluronidase activity refers to the ability to enzymatically catalyze the cleavage of hyaluronic acid. The United States Pharmacopeia (USP) XXII assay for hyaluronidase determines hyaluronidase activity indirectly by measuring the amount of higher molecular weight hyaluronic acid, or hyaluronan, (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37.degree.° C. (USP XXII-NF XVII (1990) 644-645 United States Pharmacopeia Convention, Inc, Rockville, Md.). A Reference Standard solution can be used in an assay to ascertain the relative activity, in units, of any hyaluronidase. In vitro assays to determine the hyaluronidase activity of hyaluronidases, such as PH20, including modified PH20 polypeptides, are known in the art and described herein. Exemplary assays include the microturbidity assay described herein that measures cleavage of hyaluronic acid by hyaluronidase indirectly by detecting the insoluble precipitate formed when the uncleaved hyaluronic acid binds with serum albumin. Reference Standards can be used, for example, to generate a standard curve to determine the activity in Units of the hyaluronidase being tested.

[0132](24) As used herein, neutral active refers to the ability of a PH20 polypeptide to enzymatically catalyze the cleavage of hyaluronic acid at neutral pH, such as at a pH between or about between pH 6.0 to pH 7.8.

[0133](25) As used herein, ""increased activity"" with reference to a modified PH20 hyaluronidase means that, when tested under the same conditions, the modified PH20 hyaluronidase exhibits greater hyaluronidase activity compared to an unmodified PH20 hyaluronidase not containing the amino acid replacement(s). For example, a modified PH20 hyaluronidase exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase.

[0134](26) As used herein, ""solubility"" with reference to a protein refers to a protein that is homogenous in an aqueous solution, whereby protein molecules diffuse and do not sediment spontaneously. Hence a soluble protein solution is one in which there is an absence of a visible or discrete particle in a solution containing the protein, such that the particles cannot be easily filtered. Generally, a protein is soluble if there are no visible or discrete particles in the solution.

For example, a protein is soluble if it contains no or few particles that can be removed by a filter with a pore size of 0.22 µm.~~mu.m.~~

~~[0135]~~(27) As used herein, aggregation or crystallization with reference to a protein refers to the presence of visible or discrete particles in a solution containing the protein. Typically, the particles are greater than 10 ~~.mu.m~~µm in size, such as greater than 15 ~~.mu.m~~µm, 20 ~~.mu.m~~µm, 25 ~~.mu.m~~µm, 30 ~~.mu.m~~µm, 40 ~~.mu.m~~µm, 50 ~~.mu.m~~µm or greater. Aggregation or crystallization can arise due to reduced solubility, increased denaturation of a protein or the formation of covalent bonds.

~~[0136]~~(28) As used herein, ~~"~~"denaturing condition~~" or "~~" or "denaturation condition~~"~~" refers to any condition or agent that, when exposed to a protein, affects or influences the degradation or denaturation of the protein, generally as a result of a loss or partial loss of the tertiary or secondary structure of the protein. Denaturing conditions can result in effects such as loss or reduction in activity, loss or reduction of solubility, aggregation and/or crystallization. The denaturing condition need not be one that is completely deadly to the protein, but nevertheless is one that leads to a reduction in the activity of the protein over time. Thus, a condition is denaturing if the activity of the protein is reduced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%~~)~~, 90%, 95% or more in the presence of the condition than in its absence. A denaturing condition can be due to an external stress or physical condition (e.g., agitation, temperature, time of storage, absence of a stabilizer) or can be due to the presence of a denaturing agent. For example, the denaturing condition can be caused by heat, acid or a chemical denaturant. Exemplary denaturing conditions include, but are not limited to, the presence of a strong acid or base, a concentrated inorganic salt, an organic solvent (e.g., alcohol or chloroform), urea, high or low pH (extremes of pH), elevated temperature (e.g., heat), the presence of excipients that can be denaturing (e.g., phenolic preservatives or detergent), and low or substantially no stabilizing agent that otherwise is required for stability of the protein (e.g., NaCl).

~~[0137]~~(29) As used herein, ~~"~~"denaturing agent~~" or "~~" or "denaturant~~"~~" refers to any substance, molecule or compound that causes denaturation. For example, a denaturing agent can include a strong acid or base, a concentrated inorganic salt, an organic solvent (e.g., alcohol or chloroform), a preservative, detergent or other excipient.

~~[0138]~~(30) As used herein, ~~"~~"resistance to a denaturation condition~~"~~" refers to any amount of decreased reduction or elimination of a property or activity of the protein associated with or caused by denaturation. For example, denaturation is associated with or causes increased crystallization or aggregation, reduced solubility or decreased activity. Hence, resistance to denaturation means that the protein exhibits decreased aggregation or crystallization, increased solubility or increased or greater activity (e.g., hyaluronidase activity) when exposed to a denaturing condition compared to a reference protein (e.g. unmodified enzyme). The resistance to a denaturation condition need not be absolute or permanent, but can be achieved because the denaturation of the modified hyaluronan-degrading enzyme occurs more slowly than the unmodified enzyme in the denaturation condition such that an activity or property of the modified hyaluronan-degrading enzyme is achieved for longer. For example, a modified hyaluronan-degrading enzyme, such as a modified PH20 hyaluronidase, exhibits resistance to a denaturation condition if it exhibits, for example, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, . . . 20%, . . . 30%, . . . 40%, . . . 50%, . . . 60%, . . . , 70%, . . . 80%, . . . 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99%, or 100%~~)~~ more resistance to denaturation in the presence of a denaturation condition or denaturing agent than an unmodified polypeptide. In some instances, a modified polypeptide exhibits 105%, 110%, 120%, 130%, 140%~~)~~, 150%, 200%, 300%~~)~~, 400%, 500%, or more increased resistance to denaturation compared to an unmodified polypeptide.

~~[0139]~~(31) As used herein, stability of a modified PH20 hyaluronidase means that it exhibits resistance to denaturation caused by a denaturation condition or denaturing agent. A modified PH20 polypeptide exhibits stability if it retains some activity in the presence of a denaturation condition or denaturing agent, such as at least 20%, 30%~~)~~, 40%~~)~~, 50%, 60%, 70%, 80%, 90% or more of the original or initial hyaluronidase activity prior to exposure to the denaturing condition(s). Generally, a modified PH20 hyaluronidase is stable if it retains at least 50% or more of the hyaluronidase activity under a denaturation condition compared to the absence of the denaturation condition. Assays to assess hyaluronidase activity are known to one of skill in the art and described herein. It is understood that the stability of the enzyme need not be permanent or long term, but is manifested for a duration of time in which activity is desired. For example, a modified PH20 hyaluronidase is stable if it exhibits an activity for at least 2 hours, 3 hours, 4 hours, 6 hours, 12 hours, 24 hours, one day, two days, three days, four days, five days, six days, one week, one month, six months or one year upon exposure, or during exposure, to one or more denaturing condition(s) or agent(s) (e.g., presence of a denaturing excipient such as a preservative). For example, a modified PH20 hyaluronidase is stable if it exhibits an activity upon or during exposure to one or more denaturing condition(s) or agent(s) (e.g., presence of a denaturing excipient such as a preservative) for at least 1 month at temperatures from or from about 2~~.degree.~~° C. to 8~~.degree.~~° C., inclusive or for at least 3 days at a temperature from or from about 30~~.degree.~~° C. to 42~~.degree.~~° C., inclusive.

~~[0140]~~(32) Hence, ~~"~~"stable~~"~~" or ~~"~~"stability,~~"~~" with reference to a formulation or a co-formulation provided herein, refers to one in which a modified hyaluronan-degrading enzyme, such as a modified PH20 hyaluronidase, therein is stable upon exposure to one or more denaturing condition(s) or agent(s) therein (e.g., presence of a denaturing excipient such as a preservative) for at least 1 month at temperatures from or from about 2~~.degree.~~° C. to 8~~.degree.~~° C., inclusive or for at least 3 days at a temperature from or from about 30~~.degree.~~° C. to 42~~.degree.~~° C., inclusive.

~~[0141]~~(33) As used herein, ~~"~~"increased stability~~"~~" with reference to a modified PH20 hyaluronidase means that, in the presence of the same denaturing or denaturation condition(s) (e.g., presence of a denaturing excipient such as a preservative), the modified PH20 hyaluronidase exhibits greater hyaluronidase activity compared to an unmodified PH20 hyaluronidase not containing the amino acid ~~replacements~~replacement(s). For example, a modified PH20 hyaluronidase exhibits increased stability if it exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase in the presence of a denaturing or denaturation condition(s) (e.g., in the presence of a denaturing excipient such as a preservative).

~~[0142]~~(34) As used herein, ~~"~~"elevated temperatures~~"~~" refers to temperatures that are greater than room temperature or ambient temperature. Generally, an elevated temperature is a temperature

that is at least, greater than, or about 30.degree.° C., such as 30.degree.° C. to 42.degree.° C., and generally 32.degree.° C. to 37.degree.° C. or 35.degree.° C. to 37.degree.° C., inclusive.

[0143](35) As used herein, room temperature refers to a range generally from about or at to 18.degree.° C. to about or at 32.degree.° C. Those of skill in the art appreciate that room temperature varies by location and prevailing conditions. For example, room temperatures can be higher in warmer climates such as Italy or Texas.

[0144](36) As used herein, recitation that proteins are "“compared under the same conditions"” means that different proteins are treated identically or substantially identically such that any one or more conditions that can influence the activity or properties of a protein or agent are not varied or not substantially varied between the test agents. For example, when the hyaluronidase activity of a modified PH20 polypeptide is compared to an unmodified PH20 polypeptide any one or more conditions such as the amount or concentration of the polypeptide; presence, including amount, of excipients, carriers or other components in a formulation other than the active agent (e.g., modified PH20 hyaluronidase); temperature; time of storage; storage vessel; properties of storage (e.g., agitation) and/or other conditions associated with exposure or use are identical or substantially identical between and among the compared polypeptides.

[0145](37) As used herein, "“predetermined time"” refers to a time that is established or decided in advance. For example, the predetermined time can be a time chosen in advance that is associated with the desired duration of activity of a hyaluronan-degrading enzyme depending on the desired application or use of the protein. A predetermined time can be hours, days, months or years. For example, a predetermined time can be at least about or about 2 hours, 3 hours, 4 hours, five hours, six hours, 12 hours, 24 hours, 2 days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, six months, one year or more.

[0146](38) As used herein, "“storage"” means that a formulation is not immediately administered to a subject once prepared, but is kept for a period of time under particular conditions (e.g., particular temperature; time, and/or form (e.g., liquid or lyophilized form)) prior to use. For example, a liquid formulation can be kept for days, weeks, months or years, prior to administration to a subject under varied temperatures such as refrigerated (0.degree.° C. to 10.degree.° C., such as 2.degree.° to 8.degree.° C.), room temperature (e.g., temperature up to 32.degree.° C., such as 18.degree.° C. to about or at 32.degree.° C.), or elevated temperature (e.g., 30.degree.° C. to 42.degree.° C., such as 32.degree.° C. to 37.degree.° C. or 35.degree.° C. to 37.degree.° C.).

[0147](39) As used herein, an "“excipient"” refers to a compound in a formulation of an active agent that does not provide the biological effect of the active agent when administered in the absence of the active agent. Exemplary excipients include, but are not limited to, salts, buffers, stabilizers, tonicity modifiers, metals, polymers, surfactants, preservatives, amino acids and sugars.

[0148](40) As used herein, a stabilizing agent refers to compound added to the formulation to protect the modified PH20 polypeptide or other active agent from degradation, if necessary, such as due to denaturation conditions to which a formulation herein is exposed when handled, stored or used. Thus, included are agents that prevent proteins from degradation from other components

in the compositions. Exemplary of such agents are amino acids, amino acid derivatives, amines, sugars, polyols, salts and buffers, surfactants, inhibitors or substrates and other agents as described herein.

[0149](41) As used herein, an antimicrobial effectiveness test or preservative effectiveness test (PET) demonstrates the effectiveness of the preservative system in a product. A product is inoculated with a controlled quantity of specific organisms. The test then compares the level of microorganisms found on a control sample versus the test sample over a period of 28 days. Generally, target markets have differing PET requirements. For example, the PET requirements of the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP) differ. Parameters for performing an antimicrobial effectiveness test, including in different markets, are known to one of skill in the art as described herein.

[0150](42) As used herein, an anti-microbially or anti-microbial effective amount of a preservative refers to an amount of the preservative that kills or inhibits the propagation of microbial organisms in a sample that may be introduced from storage or use. For example, for multiple-dose containers, an anti-microbially effective amount of a preservative inhibits the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses. USP and EP (EPA and EPB) have anti-microbial requirements that determine preservative effectiveness, and that vary in stringency. For example, an anti-microbial effective amount of a preservative is an amount such that at least a 1.0 log.sub.10 unit reduction in bacterial organisms occurs at 7 days following inoculation in an antimicrobial preservative effectiveness test (APET). In a particular example, an anti-microbial effective amount of a preservative is an amount such that at least a 1.0 log.sub.10 unit reduction in bacterial organisms occurs at 7 days following inoculation, at least a 3.0 log.sub.10 unit reduction of bacterial organisms occurs at 14 days following inoculation, at least no further increase in bacterial organisms occurs after 28 days following inoculation, and at least no increase in fungal organisms occurs after 7 days following inoculation. In a further example, an anti-microbial effective amount of a preservative is an amount such that at least a 1.0 log.sub.10 unit reduction of bacterial organisms occurs at 24 hours following inoculation, at least a 3.0 log.sub.10 unit reduction of bacterial organisms occurs at 7 days following inoculation, no further increase in bacterial organisms occurs after 28 days following inoculation, at least a 1.0 log.sub.10 unit reduction of fungal organisms occurs at 14 days following inoculation, and at least no further increase in fungal organisms occurs after 28 days following inoculation. In an additional example, an anti-microbial effective amount of a preservative is an amount such that at least a 2.0 log.sub.10 unit reduction of bacterial organisms occurs at 6 hours following inoculation, at least a 3.0 log.sub.10 unit reduction of bacterial organisms occurs at 24 hours following inoculation, no recovery of bacterial organisms occurs after 28 days following inoculation of the composition with the microbial inoculum, at least a 2.0 log.sub.10 unit reduction of fungal organisms occurs at 7 days following inoculation, and at least no further increase in fungal organisms occurs after 28 days following inoculation.

[0151](43) As used herein, "“preservative"” refers to a naturally occurring or synthetically or recombinantly produced substance that, when added to a molecule or protein composition, prevents microbial growth, including bacterial or fungal growth, in the composition.

[0152](44) As used herein, a "“phenolic preservative"” refers to a preservative that contains one hydroxyl group attached to an aromatic carbon ring, such as a benzene ring. Exemplary phenolic

preservatives, include but are not limited to, phenol, m-cresol, p-hydroxybenzoic acid, methylparaben, ethylparaben, and propylparaben. For example, cresols, including meta-cresol (m-cresol), has a methyl group substituted onto the benzene ring of a phenol molecule.

~~[0153]~~(45) As used herein, a ~~"~~"phenophile~~"~~" refers to a protein, such as a modified PH20 polypeptide, that exhibits stability in the presence of an anti-microbially effective amount of a preservative(s). The term ~~"~~"phenolphile~~"~~" can be used interchangeably herein with ~~"~~"phenophile~~"~~" and has the same meaning. For example, a modified PH20 polypeptide that is a phenophile or phenolphile typically exhibits increased stability compared to an unmodified PH20 hyaluronidase not containing the amino acid ~~replacements~~replacement(s) when tested under the same denaturing condition(s) containing a phenolic preservative(s). For example, a modified PH20 hyaluronidase exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%~~)~~, 180%, 190%~~)~~, 200%, 250%, 300%, 400%, 500%, 600%~~)~~, 700%, 800%, 900%, 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase in the presence of a phenolic preservative(s).

~~[0154]~~(46) As used herein, a ~~"~~"thermophile~~"~~" refers to a protein, such as a modified PH20 polypeptide, that exhibits stability under elevated temperatures greater than or about 30~~.degree.~~° C., such as 30~~.degree.~~° C. to 42~~.degree.~~° C., and generally 32~~.degree.~~° C. to 37~~.degree.~~° C. or 35~~.degree.~~° C. to 37~~.degree.~~° C. For example, a modified PH20 polypeptide that is a thermophile typically exhibits increased stability compared to an unmodified PH20 hyaluronidase not containing the amino acid replacement(s) when tested under the same elevated temperature denaturing condition(s). For example, a modified PH20 hyaluronidase exhibits at least or about at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%~~)~~, 1000% or more of the activity of the unmodified or reference PH20 hyaluronidase under elevated temperatures.

~~[0155]~~(47) As used herein, the term ~~"~~"detergent~~"~~" is used interchangeably with the term ~~"~~"surfactant~~"~~" or ~~"~~"surface acting agent.~~"~~" Surfactants are typically organic compounds that are amphiphilic, i.e., containing both hydrophobic groups (~~"~~"tails~~"~~") and hydrophilic groups (~~"~~"heads~~"~~"), which render surfactants soluble in both organic solvents and water. A surfactant can be classified by the presence of formally charged groups in its head. A non-ionic surfactant has no charge groups in its head, whereas an ionic surfactant carries a net charge in its head. A zwitterionic surfactant contains a head with two oppositely charged groups. Some examples of common surfactants include: Anionic (based on sulfate, sulfonate or carboxylate anions): perfluorooctanoate (PFOA or PFO), perfluorooctane sulfonate (PFOS), sodium dodecyl sulfate (SDS), ammonium lauryl sulfate, and other alkyl sulfate salts, sodium laureth sulfate (also known as sodium lauryl ether sulfate, or SLES), alkyl benzene sulfonate; cationic (based on quaternary ammonium cations): cetyl trimethylammonium bromide (CTAB) a.k.a. hexadecyl trimethyl ammonium bromide, and other alkyltrimethylammonium salts, cetylpyridinium chloride (CPC), polyethoxylated tallow amine (POEA), benzalkonium chloride (BAC), benzethonium chloride (BZT); Zwitterionic (amphoteric): dodecyl betaine; cocamidopropyl betaine; coco ampho glycinate; nonionic: alkyl poly(ethylene oxide), alkylphenol poly(ethylene oxide), copolymers of poly(ethylene oxide) and ~~poly(propylene~~polypropylene oxide) (commercially known as Poloxamers or Poloxamines), alkyl polyglucosides, including octyl glucoside, decyl maltoside, fatty alcohols (e.g., cetyl alcohol and oleyl alcohol), cocamide MEA,

cocamide DEA, polysorbates (Tween 20, Tween 80, etc.), Triton detergents, and dodecyl dimethylamine oxide.

[0156](48) As used herein, a "“buffer"” refers to a substance, generally a solution, that can keep its pH constant, despite the addition of strong acids or strong bases and external influences of temperature, pressure, volume or redox potential. A buffer prevents change in the concentration of another chemical substance, e.g., proton donor and acceptor systems that prevent marked changes in hydrogen ion concentration (pH). The pH values of all buffers are temperature and concentration dependent. The choice of buffer to maintain a pH value or range can be empirically determined by one of skill in the art based on the known buffering capacity of known buffers. Exemplary buffers include but are not limited to, bicarbonate buffer, cacodylate buffer, phosphate buffer or Tris buffer. For example, Tris buffer (tromethamine) is an amine based buffer that has a pKa of 8.06 and has an effective pH range between 7.9 and 9.2. For Tris buffers, pH increases about 0.03 unit per .degree.° C. temperature decrease, and decreases 0.03 to 0.05 unit per ten-fold dilution.

[0157](49) As used herein, the residues of naturally occurring .alpha. amineα-amino acids are the residues of those 20 .alpha. amineα-amino acids found in nature which are incorporated into protein by the specific recognition of the charged tRNA molecule with its cognate mRNA codon in humans.

[0158](50) As used herein, nucleic acids include DNA, RNA and analogs thereof, including peptide nucleic acids (PNA) and mixtures thereof. Nucleic acids can be single or double-stranded. When referring to probes or primers, which are optionally labeled, such as with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that their target is statistically unique or of low copy number (typically less than 5, generally less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous nucleotides of sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleic acids long.

[0159](51) As used herein, a peptide refers to a polypeptide that is from 2 to 40 amino acids in length.

[0160](52) As used herein, the amino acids which occur in the various sequences of amino acids provided herein are identified according to their known, three-letter or one-letter abbreviations (Table 1). The nucleotides which occur in the various nucleic acid fragments are designated with the standard single-letter designations used routinely in the art.

[0161](53) As used herein, an "“amino acid"” is an organic compound containing an amino group and a carboxylic acid group. A polypeptide contains two or more amino acids. For purposes herein, amino acids include the twenty naturally-occurring amino acids, non-natural amino acids and amino acid analogs (i.e., amino acids wherein the .alpha. carbonα-carbon has a side chain).

[0162](54) As used herein, "“amino acid residue"” refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described

herein are presumed to be in the "“L"” isomeric form. Residues in the "“D"” isomeric form, which are so designated, can be substituted for any L-amino acid residue as long as the desired functional property is retained by the polypeptide. NH.sub.2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in J. Biol. Chem., 243: 3557-3559 (1968), and adopted 37 C.F.R. .sctn..sctn.§§ 1.821-1.822, abbreviations for amino acid residues are shown in Table 1:

(55) TABLE-US-00001 TABLE 1 Table of Correspondence SYMBOL 1-Letter 3-Letter AMINO ACID Y Tyr Tyrosine G Gly Glycine F Phe Phenylalanine M Met Methionine A Ala Alanine S Ser Serine I Ile Isoleucine L Leu Leucine T Thr Threonine V Val Valine P Pro Proline K Lys Lysine H His Histidine Q Gln Glutamine E Glu Glutamic Acid Z Glx Glu and/or Gln W Trp Tryptophan R Arg Arginine D Asp Aspartic Acid N Asn Asparagine B Asx Asn and/or Asp C Cys Cysteine X Xaa Unknown or Other

[0163](56) It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. In addition, the phrase "“amino acid residue"” is broadly defined to include the amino acids listed in the Table of Correspondence (Table 1) and modified and unusual amino acids, such as those referred to in 37 C.F.R. .sctn..sctn.§§ 1.821-1.822, and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues, to an amino-terminal group such as NH.sub.2 or to a carboxyl-terminal group such as COOH.

[0164](57) As used herein, "“naturally occurring amino acids"” refer to the 20 L-amino acids that occur in polypeptides.

[0165](58) As used herein, "“non-natural amino acid"” refers to an organic compound that has a structure similar to a natural amino acid but has been modified structurally to mimic the structure and reactivity of a natural amino acid. Non-naturally occurring amino acids thus include, for example, amino acids or analogs of amino acids other than the 20 naturally-occurring amino acids and include, but are not limited to, the D-stereoisomers of amino acids. Exemplary non-natural amino acids are described herein and are known to those of skill in the art.

[0166](59) As used herein, an isokinetic mixture is one in which the molar ratios of amino acids has been adjusted based on their reported reaction rates (see, e.g., Ostresh et al., (1994) Biopolymers 34:1681).

[0167](60) As used herein, suitable conservative substitutions of amino acids are known to those of skill in this art and can be made generally without altering the biological activity of the resulting molecule. Those of skill in the art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. Molecular Biology of the Gene, 4th Edition, 1987, The Benjamin/Cummings Pub. co., p. 224). Such substitutions can be made in accordance with those set forth in TABLE 2 as follows:

(61) TABLE-US-00002 TABLE 2 Original residue Exemplary conservative substitution Ala (A) Gly; Ser Arg (R) Lys Asn (N) Gln; His Cys (C) Ser Gln (Q) Asn Glu (E) Asp Gly (G) Ala; Pro His (H) Asn; Gln Ile (I) Leu; Val Leu (L) Ile; Val Lys (K) Arg; Gln; Glu Met (M) Leu; Tyr; Ile Phe (F) Met; Leu; Tyr Ser (S) Thr Thr (T) Ser Trp (W) Tyr Tyr (Y) Trp; Phe Val (V) Ile; Leu

Other substitutions also are permissible and can be determined empirically or in accord with known conservative substitutions.

~~[0168]~~(62) As used herein, a DNA construct is a single or double stranded, linear or circular DNA molecule that contains segments of DNA combined and juxtaposed in a manner not found in nature. DNA constructs exist as a result of human manipulation, and include clones and other copies of manipulated molecules.

~~[0169]~~(63) As used herein, a DNA segment is a portion of a larger DNA molecule having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, which, when read from the 5′ to 3′ direction, encodes the sequence of amino acids of the specified polypeptide.

~~[0170]~~(64) As used herein, the term polynucleotide means a single- or double-stranded polymer of deoxyribonucleotides or ribonucleotide bases read from the 5′ to the 3′ end. Polynucleotides include RNA and DNA, and can be isolated from natural sources, synthesized in vitro, or prepared from a combination of natural and synthetic molecules. The length of a polynucleotide molecule is given herein in terms of nucleotides (abbreviated "~~"~~nt") or base pairs (abbreviated "~~"~~bp"). The term nucleotides is used for single- and double-stranded molecules where the context permits. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term base pairs. It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide can differ slightly in length and that the ends thereof can be staggered; thus all nucleotides within a double-stranded polynucleotide molecule cannot be paired. Such unpaired ends will, in general, not exceed 20 nucleotides in length.

~~[0171]~~(65) As used herein, "~~"~~at a position corresponding to~~"~~" or recitation that nucleotides or amino acid positions "~~"~~correspond to~~"~~" nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm, such as the GAP algorithm. For purposes herein, alignment of a PH20 sequence is to the amino acid sequence set forth in any of SEQ ID NOS: 3, 7 or 32-66, and in particular SEQ ID NO:3. Hence, reference herein that a position or amino acid replacement corresponds to positions with reference to SEQ ID NO:3 also means that the position or amino acid replacement corresponds to positions with reference to any of SEQ ID NOS: 7 or 32-66, since the sequences therein are identical to the corresponding residues as set forth in SEQ ID NO:3. By aligning the sequences, one skilled in the art can identify corresponding residues, for example, using conserved and identical amino acid residues as guides. In general, to identify corresponding positions, the sequences of amino acids are aligned so that the highest order match is obtained (see, e.g.: Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M.,

and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; Carrillo et al. (1988) SIAM J Applied Math 48:1073). FIG. 2 (A-L) exemplifies exemplary alignments and identification of exemplary corresponding residues for replacement.

~~[0172]~~(66) As used herein, ~~"~~"sequence identity~~"~~" refers to the number of identical or similar amino acids or nucleotide bases in a comparison between a test and a reference polypeptide or polynucleotide. Sequence identity can be determined by sequence alignment of nucleic acid or protein sequences to identify regions of similarity or identity. For purposes herein, sequence identity is generally determined by alignment to identify identical residues. Alignment can be local or global, but for purposes herein alignment is generally a global alignment where the full-length of each sequence is compared. Matches, mismatches and gaps can be identified between compared sequences. Gaps are null amino acids or nucleotides inserted between the residues of aligned sequences so that identical or similar characters are aligned. Generally, there can be internal and terminal gaps. Sequence identity can be determined by taking into account gaps as the number of identical residues/length of the shortest sequence~~.times.~~×100. When using gap penalties, sequence identity can be determined with no penalty for end gaps (e.g., terminal gaps are not penalized). Alternatively, sequence identity can be determined without taking into account gaps as the number of identical positions/length of the total aligned sequence~~.times.~~×100.

~~[0173]~~(67) As used herein, a ~~"~~"global alignment~~"~~" is an alignment that aligns two sequences from beginning to end, aligning each letter in each sequence only once. An alignment is produced, regardless of whether or not there is similarity or identity between the sequences. For example, 50% sequence identity based on ~~"~~"global alignment~~"~~" means that in an alignment of the full sequence of two compared sequences each of 100 nucleotides in length, 50% of the residues are the same. It is understood that global alignment also can be used in determining sequence identity even when the length of the aligned sequences is not the same. The differences in the terminal ends of the sequences will be taken into account in determining sequence identity, unless the ~~"~~"no penalty for end gaps~~"~~" is selected. Generally, a global alignment is used on sequences that share significant similarity over most of their length. Exemplary algorithms for performing global alignment include the Needleman-Wunsch algorithm (Needleman et al. J. Mol. Biol. 48: 443 (1970). Exemplary programs for performing global alignment are publicly available and include the Global Sequence Alignment Tool available at the National Center for Biotechnology Information (NCBI) website (ncbi.nlm.nih.gov/), and the program available at deepc2.psi.iastate.edu/aat/align/align.html.

~~[0174]~~(68) As used herein, a ~~"~~"local alignment~~"~~" is an alignment that aligns two sequence, but only aligns those portions of the sequences that share similarity or identity. Hence, a local alignment determines if sub-segments of one sequence are present in another sequence. If there is no similarity, no alignment will be returned. Local alignment algorithms include BLAST or Smith-Waterman algorithm (Adv. Appl. Math. 2: 482 (1981)). For example, 50% sequence identity based on ~~"~~"local alignment~~"~~" means that in an alignment of the full sequence of two compared sequences of any length, a region of similarity or identity of 100 nucleotides in length has 50% of the residues that are the same in the region of similarity or identity.

[0175](69) For purposes herein, sequence identity can be determined by standard alignment algorithm programs used with default gap penalties established by each supplier. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non identities) and the weighted comparison matrix of Gribskov et al. Nucl. Acids Res. 14: 6745 (1986), as described by Schwartz and Dayhoff, eds., Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Whether any two nucleic acid molecules have nucleotide sequences or any two polypeptides have amino acid sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical," or other similar variations reciting a percent identity, can be determined using known computer algorithms based on local or global alignment (see e.g., wikipedia.org/wiki/Sequence_alignment_software, providing links to dozens of known and publicly available alignment databases and programs). Generally, for purposes herein sequence identity is determined using computer algorithms based on global alignment, such as the Needleman-Wunsch Global Sequence Alignment tool available from NCBI/BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&Page_TYPE=BlastHome); LAlign (William Pearson implementing the Huang and Miller algorithm (Adv. Appl. Math. (1991) 12:337-357)); and program from Xiaoqui Huang available at deepc2.psi.iastate.edu/aat/align/align.html. Generally, when comparing nucleotide sequences herein, an alignment with penalty for end gaps is used. Local alignment also can be used when the sequences being compared are substantially the same length.

[0176](70) Therefore, as used herein, the term "identity" represents a comparison or alignment between a test and a reference polypeptide or polynucleotide. In one non-limiting example, "at least 90% identical to" refers to percent identities from 90 to 100% relative to the reference polypeptide or polynucleotide. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide or polynucleotide length of 100 amino acids or nucleotides are compared, no more than 10% (i.e., 10 out of 100) of amino acids or nucleotides in the test polypeptide or polynucleotide differs from that of the reference polypeptides. Similar comparisons can be made between a test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more locations of varying length up to the maximum allowable, e.g., 10/100 amino acid difference (approximately 90% identity). Differences also can be due to deletions or truncations of amino acid residues. Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. Depending on the length of the compared sequences, at the level of homologies or identities above about 85-90%, the result can be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.

[0177](71) As used herein, an allelic variant or allelic variation references any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and can result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or can encode polypeptides having altered amino acid sequence. The term "allelic variant" also is used herein to denote a protein encoded by an allelic variant of a gene. Typically the reference form of the gene encodes a wildtype form and/or predominant form of a polypeptide from a population or single reference member of a species. Typically, allelic variants, which include variants between and among

species typically have at least 80%, 90% or greater amino acid identity with a wildtype and/or predominant form from the same species; the degree of identity depends upon the gene and whether comparison is interspecies or intraspecies. Generally, intraspecies allelic variants have at least about 80%, 85%, 90% or 95% identity or greater with a wildtype and/or predominant form, including 96%, 97%, 98%, 99% or greater identity with a wildtype and/or predominant form of a polypeptide. Reference to an allelic variant herein generally refers to variations in proteins among members of the same species.

[0178](72) As used herein, "“allele,"” which is used interchangeably herein with "“allelic variant"” refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for that gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide or several nucleotides, and can include modifications such as substitutions, deletions and insertions of nucleotides. An allele of a gene also can be a form of a gene containing a mutation.

[0179](73) As used herein, species variants refer to variants in polypeptides among different species, including different mammalian species, such as mouse and human. Exemplary of species variants provided herein are primate PH20, such as, but not limited to, human, chimpanzee, macaque, cynomolgus monkey, gibbon, orangutan, or marmoset. Generally, species variants have 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity. Corresponding residues between and among species variants can be determined by comparing and aligning sequences to maximize the number of matching nucleotides or residues, for example, such that identity between the sequences is equal to or greater than 95%, equal to or greater than 96%, equal to or greater than 97%, equal to or greater than 98% or equal to greater than 99%. The position of interest is then given the number assigned in the reference nucleic acid molecule. Alignment can be effected manually or by eye, particularly where sequence identity is greater than 80%.

[0180](74) As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities, as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound can, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

[0181](75) As used herein, isolated or purified polypeptide or protein or biologically-active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. Preparations can be determined to be substantially free if they appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or

sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound, however, can be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

~~[0182]~~(76) Hence, reference to a substantially purified polypeptide, such as a substantially purified PH20 polypeptide refers to preparations of PH20 proteins that are substantially free of cellular material, includes preparations of proteins in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly-produced. In one embodiment, the term substantially free of cellular material includes preparations of enzyme proteins having less than about 30% (by dry weight) of non-enzyme proteins (also referred to herein as contaminating proteins), generally less than about 20% of non-enzyme proteins or 10% of non-enzyme proteins or less than about 5% of non-enzyme proteins. When the enzyme protein is recombinantly produced, it also is substantially free of culture medium, i.e., culture medium represents less than about or at 20%, 10% or 5% of the volume of the enzyme protein preparation.

~~[0183]~~(77) As used herein, the term substantially free of chemical precursors or other chemicals includes preparations of enzyme proteins in which the protein is separated from chemical precursors or other chemicals that are involved in the synthesis of the protein. The term includes preparations of enzyme proteins having less than about 30% (by dry weight), 20%, 10%, 5% or less of chemical precursors or non-enzyme chemicals or components.

~~[0184]~~(78) As used herein, synthetic, with reference to, for example, a synthetic nucleic acid molecule or a synthetic gene or a synthetic peptide refers to a nucleic acid molecule or polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods.

~~[0185]~~(79) As used herein, production by recombinant means or using recombinant DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

~~[0186]~~(80) As used herein, vector (or plasmid) refers to discrete elements that are used to introduce a heterologous nucleic acid into cells for either expression or replication thereof. The vectors typically remain episomal, but can be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well known to those of skill in the art.

~~[0187]~~(81) As used herein, an expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Such additional segments can include promoter and terminator sequences, and optionally can include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both. Thus, an

expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

[0188](82) As used herein, vector also includes "“virus vectors"” or "“viral vectors."” Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells. Viral vectors include, but are not limited to, adenoviral vectors, retroviral vectors and vaccinia virus vectors.

[0189](83) As used herein, "“operably" or "” or “operatively linked"” when referring to DNA segments means that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates downstream of the promoter and upstream of any transcribed sequences. The promoter is usually the domain to which the transcriptional machinery binds to initiate transcription and proceeds through the coding segment to the terminator.

[0190](84) As used herein, a conjugate refers to a modified PH20 polypeptide linked directly or indirectly to one or more other polypeptides or chemical moieties. Such conjugates include fusion proteins, those produced by chemical conjugates and those produced by any other method whereby at least one modified PH20 polypeptide is linked, directly or indirectly to another polypeptide or chemical moiety so long as the conjugate retains hyaluronidase activity. Exemplary of conjugates provided herein include PH20 polypeptides linked directly or indirectly to a multimerization domain (e. g. an Fc moiety), a toxin, a label or a drug.

[0191](85) As used herein, a fusion protein refers to a polypeptide encoded by a nucleic acid sequence containing a coding sequence from one nucleic acid molecule and the coding sequence from another nucleic acid molecule in which the coding sequences are in the same reading frame such that when the fusion construct is transcribed and translated in a host cell, the protein is produced containing the two proteins. The two molecules can be adjacent in the construct or separated by a linker polypeptide that contains, 1, 2, 3, or more, but typically fewer than 10, 9, 8, 7, or 6 amino acids. The protein product encoded by a fusion construct is referred to as a fusion polypeptide. Examples of fusion polypeptides include Fc fusions.

[0192](86) As used herein, a polymer that is conjugated to a modified PH20 polypeptide refers to any polymer that is covalently or otherwise stably linked, directly or via a linker, to such polypeptide. Such polymers, typically increase serum half-life, and include, but are not limited to, sialic moieties, polyethylene glycol (PEG) moieties, dextran, and sugar and other moieties, such as for glycosylation.

[0193](87) As used herein, the term assessing or determining is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the activity of a product, and also of obtaining an index, ratio, percentage, visual or other value indicative of the level of the activity. Assessment can be direct or indirect.

[0194](88) As used herein, a "“composition"” refers to any mixture of two or more products or compounds. It can be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous, or any combination thereof.

[0195](89) As used herein, a formulation refers to a composition containing at least one active pharmaceutical or therapeutic agent and one or more excipients.

[0196](90) As used herein, a co-formulation refers to a composition containing two or more active or pharmaceutical or therapeutic agents and one or more excipients. For example, a co-formulation of a fast-acting insulin and a hyaluronan degrading enzyme contains a fast-acting insulin, a hyaluronan degrading enzyme, and one or more excipients.

[0197](91) As used herein, "“a combination"” refers to any association between two or among more items or elements. Exemplary combinations include, but are not limited to, two or more pharmaceutical compositions, a composition containing two or more active ingredients, such as two modified PH20 polypeptidepolypeptides; a modified PH20 polypeptide and an anticancer agent, such as a chemotherapeutic compound; a modified PH20 polypeptide and a therapeutic agent (e.g. an insulin); a modified PH20 polypeptide and a plurality therapeutic and/or imaging agents, or any association thereof. Such combinations can be packaged as kits.

[0198](92) As used herein, a kit is a packaged combination, optionally, including instructions for use of the combination and/or other reactions and components for such use.

[0199](93) As used herein, "“disease or disorder"” refers to a pathological condition in an organism resulting from cause or condition including, but not limited to, infections, acquired conditions, genetic conditions, and characterized by identifiable symptoms.

[0200](94) As used herein, a hyaluronan-associated disease, disorder or condition refers to any disease or condition in which hyaluronan levels are elevated as cause, consequence or otherwise observed in the disease or condition. Hyaluronan-associated diseases and conditions are associated with elevated hyaluronan expression in a tissue or cell, increased interstitial fluid pressure, decreased vascular volume, and/or increased water content in a tissue. Hyaluronan-associated diseases, disorders or conditions can be treated by administration of a composition containing a hyaluronan degrading enzyme, such as a hyaluronidase, for example, a soluble hyaluronidase, either alone or in combination with or in addition to another treatment and/or agent. Exemplary diseases and conditions, include, but are not limited to, hyaluronan-rich cancers, for example, tumors, including solid tumors such as late-stage cancers, metastatic cancers, undifferentiated cancers, ovarian cancer, in situ carcinoma (ISC), squamous cell carcinoma (SCC), prostate cancer, pancreatic cancer, non-small cell lung cancer, breast cancer, colon cancer and other cancers. Exemplary hyaluronan-associated diseases and conditions also are diseases that are associated with elevated interstitial fluid pressure, such as diseases associated with disc pressure, and edema, for example, edema caused by organ transplant, stroke, brain trauma or other injury. Exemplary hyaluronan-associated diseases and conditions include diseases and conditions associated with elevated interstitial fluid pressure, decreased vascular volume, and/or increased water content in a tissue, including cancers, disc pressure and edema. In one example, treatment of the hyaluronan-associated condition, disease or disorder includes

amelioration, reduction, or other beneficial effect on one or more of increased interstitial fluid pressure (IFP), decreased vascular volume, and increased water content in a tissue.

[0201](95) As used herein, "“treating"” a subject with a disease or condition means that the subject's symptoms are partially or totally alleviated, or remain static following treatment. Hence treatment encompasses prophylaxis, therapy and/or cure. Prophylaxis refers to prevention of a potential disease and/or a prevention of worsening of symptoms or progression of a disease. Treatment also encompasses any pharmaceutical use of a modified interferon and compositions provided herein.

[0202](96) As used herein, a pharmaceutically effective agent or therapeutic agent includes any bioactive agent that can exhibit a therapeutic effect to treat a disease or disorder. Exemplary therapeutic agents are described herein. Therapeutic agents include, but are not limited to, anesthetics, vasoconstrictors, dispersing agents, conventional therapeutic drugs, including small molecule drugs, including, but not limited to, bisphosphonates, and therapeutic proteins, including, but not limited to, insulin, IgG molecules, antibodies, cytokines and coagulation factors.

[0203](97) As used herein, "“insulin"” refers to a hormone, precursor or a synthetic or recombinant analog thereof that acts to increase glucose uptake and storage and/or decrease endogenous glucose production. Insulin and analogs thereof are well known to one of skill in the art, including in human and allelic and species variants thereof. Insulin is translated as a precursor polypeptide designated preproinsulin (110 amino acid for human insulin), containing a signal peptide that directs the protein to the endoplasmic reticulum (ER) wherein the signal sequence is cleaved, resulting in proinsulin. Proinsulin is processed further to release a C- or connecting chain peptide (a 31 amino acid C-chain in human insulin). The resulting insulin contains an A-chain (21 amino acid in length in human insulin; set forth in SEQ ID NO:862) and a B-chain (30 amino acid in length in human insulin; set forth in SEQ ID NO:863) which are cross-linked by disulfide bonds. A fully cross-linked human insulin contains three disulfide bridges: one between position 7 of the A-chain and position 7 of the B-chain, a second between position 20 of the A-chain and position 19 of the B-chain, and a third between positions 6 and 11 of the A-chain. Reference to an insulin includes monomeric and multimeric insulins, including hexameric insulins, as well as humanized insulins. Exemplary insulin polypeptides are those of mammalian, including human, origin. Reference to insulin includes preproinsulin, proinsulin and insulin polypeptides in single-chain or two-chain forms, truncated forms thereof that have activity, and includes allelic variants and species variants of human insulin, variants encoded by splice variants, and other variants, such as insulin analogs. An exemplary insulin is human insulin having a sequence of amino acids of the A- and B-chains of human insulin are set forth in SEQ ID NOS: 862 and 863, respectively, and variants or analogs thereof that exhibit at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity thereto to one or both of the A-chain or B-chain and that acts to increase glucose uptake and storage and/or decrease endogenous glucose production. A further exemplary insulin is porcine insulin having a sequence of amino acids for the preproinsulin as set forth in SEQ ID NO:864, whereby the A chain corresponds to amino acid residue positions 88-108 and the B-chain correspond to amino acid, and variants or analogs thereof that exhibit at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity thereto to one or both of the A-chain

or B-chain and that acts to increase glucose uptake and storage and/or decrease endogenous glucose production.

[0204](98) As used herein, "“fast-acting insulin"” refers to any insulin that exhibits peak insulin levels at or about not more than four hours following subcutaneous administration to a subject. Fast-acting insulins include any insulin or any fast-acting insulin composition for acute administration to a diabetic subject in response to an actual, perceived, or anticipated hyperglycemic condition in the subject arising at the time of, or within about four hours following, administration of the fast-acting insulin (such as a prandial hyperglycemic condition resulting or anticipated to result from, consumption of a meal), whereby the fast-acting insulin is able to prevent, control or ameliorate the acute hyperglycemic condition. Fast-acting insulins include recombinant insulins and isolated insulins (also referred to as "“regular"” insulins) such as the insulin sold as human insulin, porcine insulins and bovine insulins, as well as rapid acting insulin analogs (also termed fast-acting insulin analogs herein) designed to be rapid acting by virtue of amino acid changes. Exemplary regular insulin preparations include, but are not limited to, human regular insulins, such as those sold under the trademarks Humulin.RTM.® R, Novolin.RTM.® R and Velosulin.RTM.®, Insulin Human, USP and Insulin Human Injection, USP, as well as acid formulations of insulin, such as, for example, Toronto Insulin, Old Insulin, and Clear Insulin, and regular pig insulins, such as Iletin II.RTM.® (porcine insulin). Regular insulins typically have an onset of action of between 30 minutes to an hour, and a peak insulin level of 2-5 hours post administration.

[0205](99) As used herein, rapid acting insulin analogs (also called fast-acting insulin analogs) are insulins that have a rapid onset of action. Rapid insulins typically are insulin analogs that have been engineered, such as by the introduction of one or more amino acid substitutions, to be more rapid acting than regular insulins. Rapid acting insulin analogs typically have an onset of action of 10-30 minutes post injection, with peak insulin levels observed 30-90 minutes post injection. Exemplary rapid acting insulin analogs are analogs of human insulin containing one or more amino acid changes in the A-chain and/or B-chain of human insulin set forth in SEQ ID NO:862 or 863, respectively, and that exhibit an onset of action 10-30 minutes post injection with peak insulin levels observed 30-90 minutes post injection. Exemplary rapid acting insulin analogs include, but are not limited to, for example, insulin lispro (e.g., Humalog.RTM.® insulin), insulin aspart (e.g., NovoLog.RTM.® insulin), and insulin glulisine (e.g., Apidra.RTM.® insulin) the fast-acting insulin composition sold as VIAject.RTM.® and VIAtab.RTM.® (see, e.g., U.S. Pat. No. 7,279,457). The amino acid sequence of exemplary rapid acting insulin analogs have an A chain with a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in any of SEQ ID NOS:865-867. Also included are any other insulins that have an onset of action of 30 minutes or less and a peak level before 90 minutes, typically 30-90 minutes, post injection.

[0206](100) As used herein, a human insulin refers to an insulin that is synthetic or recombinantly produced based upon the human polypeptide, including allelic variants and analogs thereof.

[0207](101) As used herein, fast-acting human insulins or human fast-acting insulin compositions include any human insulin or composition of a human insulin that is fast-acting, but excludes non-human insulins, such as regular pig insulin.

[0208](102) As used herein, the terms "“basal-acting insulins,"” or "“basal insulins"” refer to insulins administered to maintain a basal insulin level as part of an overall treatment regimen for treating a chronic condition such diabetes. Typically, a basal-acting insulin is formulated to maintain an approximately steady state insulin level by the controlled release of insulin when administered periodically (e.g., once or twice daily). Basal-acting insulins include crystalline insulins (e.g., NPH and Lente RTM.®, protamine insulin, surfen insulin), basal insulin analogs (insulin glargine, HOE 901, NovoSol Basal) and other chemical formulations of insulin (e.g., gum arabic, lecithin or oil suspensions) that retard the absorption rate of regular insulin. As used herein, the basal-acting insulins can include insulins that are typically understood as long-acting (typically reaching a relatively low peak concentration, while having a maximum duration of action over about 20-30 hours) or intermediate-acting (typically causing peak insulin concentrations at about 4-12 hours after administration).

[0209](103) As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease or other indication, are ameliorated or otherwise beneficially altered.

[0210](104) As used herein, therapeutic effect means an effect resulting from treatment of a subject that alters, typically improves or ameliorates the symptoms of a disease or condition or that cures a disease or condition. A therapeutically effective amount refers to the amount of a composition, molecule or compound which results in a therapeutic effect following administration to a subject.

[0211](105) As used herein, the term "“subject"” refers to an animal, including a mammal, such as a human being.

[0212](106) As used herein, a patient refers to a human subject exhibiting symptoms of a disease or disorder.

[0213](107) As used herein, amelioration of the symptoms of a particular disease or disorder by a treatment, such as by administration of a pharmaceutical composition or other therapeutic, refers to any lessening, whether permanent or temporary, lasting or transient, of the symptoms that can be attributed to or associated with administration of the composition or therapeutic.

[0214](108) As used herein, prevention or prophylaxis refers to methods in which the risk of developing a disease or condition is reduced.

[0215](109) As used herein, a "“therapeutically effective amount"” or a "“therapeutically effective dose"” refers to the quantity of an agent, compound, material, or composition containing a compound that is at least sufficient to produce a therapeutic effect. Hence, it is the quantity necessary for preventing, curing, ameliorating, arresting or partially arresting a symptom of a disease or disorder.

[0216](110) As used herein, unit dose form refers to physically discrete units suitable for human and animal subjects and packaged individually as is known in the art.

[0217](111) As used herein, a single dosage formulation refers to a formulation containing a single dose of therapeutic agent for direct administration. Single dosage formulations generally do not contain any preservatives.

[0218](112) As used herein, a multi-dose formulation refers to a formulation that contains multiple doses of a therapeutic agent and that can be directly administered to provide several single doses of the therapeutic agent. The doses can be administered over the course of minutes, hours, weeks, days or months. Multidose formulations can allow dose adjustment, dose-pooling and/or dose-splitting. Because multi-dose formulations are used over time, they generally contain one or more preservatives to prevent microbial growth.

[0219](113) As used herein, an "“article of manufacture”" is a product that is made and sold. As used throughout this application, the term is intended to encompass a therapeutic agent with a soluble PH20, such as esPH20, or an esPH20 alone, contained in the same or separate articles of packaging.

[0220](114) As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

[0221](115) As used herein, a "“control”" or "“standard”" refers to a sample that is substantially identical to the test sample, except that it is not treated with a test parameter, or, if it is a plasma sample, it can be from a normal volunteer not affected with the condition of interest. A control also can be an internal control. For example, a control can be a sample, such as a virus, that has a known property or activity.

[0222](116) As used herein, the singular forms "“a,” "” “an”" and "“the”" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "“an”" agent includes one or more agents.

[0223](117) As used herein, the term "“or”" is used to mean "“and/or”" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

[0224](118) As used herein, ranges and amounts can be expressed as "“about”" a particular value or range. About also includes the exact amount. Hence "“about 5 bases”" means "“about 5 bases”" and also "“5 bases.”"

[0225](119) As used herein, "“optional" or "” or “optionally”" means that the subsequently described event or circumstance does or does not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally substituted group means that the group is unsubstituted or is substituted.

[0226](120) As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) Biochem. 11:1726).

[0227](121) For clarity of disclosure, and not by way of limitation, the detailed description is divided into the subsections that follow.

(122) B. PH20 Hyaluronidase

[0228](123) Provided herein are modified PH20 polypeptides. PH20 (also known as sperm surface protein, sperm adhesion molecule 1 or SPAM1) is a hyaluronidase that hydrolyzes hyaluronan (also called hyaluronic acid, hyaluronate or HA) found in connective tissues such as the extracellular matrix. Hyaluronan polymers are composed of repeating disaccharide units, D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc), linked together via alternating .beta.β-1.fwdarw.4 and .beta.β-1.fwdarw.3 glycosidic bonds. Hyaluronan chains can reach about 25,000 disaccharide repeats or more in length, and polymers of hyaluronan can range in size from about 5,000 to 20,000,000 Da in vivo. Hyaluronan, also called hyaluronic acid or hyaluronate, is a non-sulfated glycosaminoglycan that is widely distributed throughout connective, epithelial, and neural tissues. Hyaluronan is an essential component of the extracellular matrix and a major constituent of the interstitial barrier. PH20 is an endo-.beta.-N-acetyl-hexosaminidaseendo-β-N-acetyl-hexosaminidase that hydrolyzes the .beta.1β1.fwdarw.4 glycosidic bond of hyaluronic acid into various oligosaccharide lengths such as tetrasaccharides and hexasaccharides. PH20 has both hydrolytic and transglycosidase activities. In addition to degrading hyaluronic acid, PH20 also can degrade chondroitin sulfates, such as C4-S and C6-S. PH20 can exhibit hyaluronidase activity at acidic pH and neutral pH.

[0229](124) 1. Structure

[0230](125) PH20 cDNA has been cloned from numerous mammalian species. Exemplary PH20 precursor polypeptides include, but are not limited to, human (SEQ ID NO:6), bovine (SEQ ID NOS:15 or 17), rabbit (SEQ ID NO:23), Cynomolgus monkey (SEQ ID NO:13), guinea pig (SEQ ID NO:28), rat (SEQ ID NO:21), mouse (SEQ ID NO:19), chimpanzee (SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:869) Rhesus monkey (SEQ ID NO:11), Fox (SEQ ID NO:30), Gibbon (SEQ ID NO:856), Marmoset (SEQ ID NO:858) or orangutan (SEQ ID NO:860) PH20 polypeptides. The mRNA transcript is typically translated to generate a precursor protein containing a 35 amino acid signal sequence at the N-terminus. Following transport to the ER, the signal peptide is removed to yield a mature PH20 polypeptide. Exemplary mature PH20 polypeptides include, but are not limited to, human (SEQ ID NO:7), bovine (SEQ ID NOS:16 or 18), rabbit (SEQ ID NO:24), Cynomolgus monkey (SEQ ID NO:14), guinea pig (SEQ ID NO:29), rat (SEQ ID NO:22), mouse (SEQ ID NO:20), chimpanzee (SEQ ID NO:10 or SEQ ID NO:870), Rhesus monkey (SEQ ID NO:12), Fox (SEQ ID NO:31), Gibbon (SEQ ID NO:857), Marmoset (SEQ ID NO:859) or orangutan (SEQ ID NO:861) PH20 polypeptides. For example, the human PH20 mRNA transcript is normally translated to generate a 509 amino acid precursor protein (SEQ ID NO:6) containing a 35 amino acid signal sequence at the N-terminus (amino acid residue positions 1-35 of SEQ ID NO:6). Thus, following transport to the ER and removal of the signal peptide, a 474 amino acid mature polypeptide with an amino acid sequence set forth in SEQ ID NO:7 is produced. Sequences of PH20 from ovine are also known (see e.g., SEQ ID NOS: 25-27).

[0231](126) In particular, human PH20 has the sequence of amino acids set forth in SEQ ID NO:6. The mature human PH20 lacking a signal sequence is set forth in SEQ ID NO:7. Allelic variants and other variants of PH20 are known. Other sequences of PH20 have been reported. For example, a PH20 variant is known as set forth in the precursor sequence set forth in SEQ ID NO:68 that contains an Ala at position 48 and a Trp at position 499, or the mature sequence thereof set forth in SEQ ID NO:69 containing the corresponding differences at positions 13 and 464, respectively, compared to the sequence set forth in SEQ ID NO:7 (see e.g., Gmachl et al.

(1993) FEBS Lett., 336:545-548; GenBank Accession No. AAC60607). Further, a natural variant of PH20 has been identified containing a Glutamine (Gln; Q) at position 5 as compared to the precursor sequence of amino acids set forth in SEQ ID NO:6 (see e.g., SEQ ID NO:70, see also Varela et al. (2011) Nature, 469:539-542). Another natural variant contains an Alanine (Ala; A) at position 47 compared to the sequence of amino acids set forth in SEQ ID NO:6 (as set forth in SEQ ID NO: 71) and corresponding to position 12 compared to the sequence of amino acids set forth in SEQ ID NO: 3 or 7 (as set forth in SEQ ID NO:72).

[0232](127) The sequence and structure of PH20 polypeptides is highly conserved. Sequence identity between and among PH20 proteins from various species is about 50% to 90%. The hydrophobic N-terminal signal sequence of 35 amino acids in length is generally conserved among PH20 hyaluronidase polypeptides. PH20 hyaluronidases contain a common core hyaluronidase domain region of about 340 amino acids in length that corresponds to amino acid residues 38-374 of the precursor human PH20 sequence set forth in SEQ ID NO:6. A mature PH20 polypeptide lacking the signal sequence and containing a contiguous sequence of amino acids having a C-terminal amino acid residue corresponding to amino acid residue 464 of SEQ ID NO:6 (e.g., amino acid residues corresponding to positions 36-464 of the amino acid sequence set forth in SEQ ID NO:6) is the minimal sequence required for hyaluronidase activity (see e.g., U.S. patent application Ser. No. 10/795,095, which is issued as U.S. Pat. No. 7,767,429; see also U.S. Publication No. US20100143457).

[0233](128) Within the common hyaluronidase domain region, at least 57 amino acids are conserved between and among species (see e.g., Arming et al. (1997) Eur. J. Biochem., 247:810-814; ten Have et al. (1998) Reprod. Fertil. Dev., 10:165-72; Chowpongpang et al. (2004) Biotechnology Letters, 26:1247-1252). For example, PH20 hyaluronidases contain 12 conserved cysteine residues corresponding to amino acid residue 25, 189, 203, 316, 341, 346, 352, 400, 402, 408, 423 and 429 of the sequence of amino acids of a mature PH20 lacking the signal sequence such as set forth in SEQ ID NO: 3 or 7 (corresponding to amino acid residues 60, 224, 238, 351, 376, 381, 387, 435, 437, 443, 458 or 464 of full-length human PH20 set forth in SEQ ID NO:6). Cysteine residues corresponding to 25 and 316 and cysteine residues corresponding to 189 and 203 form disulfide bridges. The other cysteine residues also form disulfide bridges, are involved in posttranslational protein maturation and/or in activity modulation. For example, further four disulfide bonds are formed between the cysteine residues C376 and C387; between C381 and C435; between C437 and C443; and between C458 and C464 of the polypeptide exemplified in SEQ ID NO:6 (corresponding to positions C341 and C352; between C346 and C400; between C402 and C408; and between C423 and C429 of the mature polypeptide set forth in SEQ ID NO:3 or 7, respectively).

[0234](129) Amino acid residues corresponding to amino acid residue D111, E113 and E249 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 are acidic residues part of the enzyme active site and are conserved between and among PH20 species. Amino acid residues R176, R246, R252 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 are also conserved between and among species and contribute to substrate binding and/or hyaluronidase activity. Amino acid mutations D111N, E113Q, R176G, E249N and R252T result in enzymes that have no detectable enzymatic activity or residual enzymatic activity (see e.g., Arming et al. (1997) Eur. J. Biochem., 247:810-814).

[0235](130) The results herein confirm the requirement of PH20 amino acid residues corresponding to positions 25, 111, 113, 176, 189, 203, 246, 249, 252, 316, 341, 346, 352, 400, 402, 408, 423 and 429 of the sequence of amino acids set forth in a mature PH20 lacking the signal sequence such as set forth in SEQ ID NO: 3 or 7 for hyaluronidase activity, since mutagenesis of these residues results in an enzyme that is not active (e.g., it is not expressed or is inactive when expressed, see e.g., Tables 5 and 10). The exception is that amino acid replacement corresponding to R176K and C316D resulted in mutants that generated some residual hyaluronidase activity.

[0236](131) Glycosylation also is required for PH20 hyaluronidase activity based on the recognition motif NxSN×S or NxTN×T. There are six N-linked oligosaccharides at amino acid residues corresponding to positions N47, N131, N200, N219, N333 and N358 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 (corresponding to amino acid residues N82, N166, N235, N254, N368 and N393 of human PH20 set forth in SEQ ID NO: 6). In particular, at least N-linked glycosylation sites corresponding to amino acid residues N200, N333 and N358 are required for secretion and/or activity of the enzyme (see e.g., U.S. Publication No. US20100143457). For example, a PH20 polypeptide containing amino acid mutations N200A, N333A, N358A or N333A/N393A result in inactive proteins. Single mutations of glycosylation sites N47A, N131A, N219A, N47A/N131A, N47A/N219A, N131A/N291A retain activity. The N-linked glycosylation site corresponding to amino acid residue N368 of human PH20 set forth in SEQ ID NO:6 is conserved between and among species (see e.g., Chowpongpang et al. (2004) Biotechnology Letters, 26:1247-1252). PH20 hyaluronidases also contains O-linked glycosylation sites. For example, human PH20 has one O-linked oligosaccharide at the amino acid residue corresponding to amino acid T440 of the sequence of amino acids set forth in SEQ ID NO:3 or 7 (corresponding to amino acid residue T475 in SEQ ID NO:6).

[0237](132) In addition to the catalytic sites, PH20 also contains a hyaluronan-binding site. This site is located in the Peptide 2 region, which corresponds to amino acid positions 205-235 of the precursor polypeptide set forth in SEQ ID NO:6 and positions 170-200 of the mature polypeptide set forth in SEQ ID NO:3 or 7. This region is highly conserved among hyaluronidases and is similar to the heparin binding motif. Mutation of the arginine residue at position 176 (corresponding to the mature PH20 polypeptide set forth in SEQ ID NO:3 or 7) to a glycine results in a polypeptide with only about 1% of the hyaluronidase activity of the wild type polypeptide (Arming et al., (1997) Eur. J. Biochem. 247:810-814).

[0238](133) PH20 polypeptides contain a glycosyl phosphatidylinositol (GPI) anchor attached to the C-terminus of the protein that anchors the protein to the extracellular leaflet of the plasma membrane of cells. At least human, monkey, mouse and guinea pig PH20 are strongly attached to the plasma membrane via the GPI anchor, which can be released by treating with phosphatidylinositol-specific phospholipase C (PI-PLC; see e.g., Lin et al. (1994) Journal of Cell Biology, 125:1157-1163; Lin et al. (1993) Proc. Natl. Acad. Sci., 90:10071-10075). Other PH20 enzymes, such as bovine PH20, are loosely attached to the plasma membrane and are not anchored via a phospholipase sensitive anchor. As discussed below, soluble active forms that, when expressed, are not attached to the membrane but are secreted can be generated by removal of all of a portion of the GPI anchor attachment signal site (see also U.S. Pat. No. 7,767,429; U.S. Publication No. US20100143457). These include, for example, soluble PH20 polypeptides

set forth in any of SEQ ID NOS: 3 or 32-66, or precursor forms thereof containing a signal sequence.

[0239](134) GPI-anchored proteins, for example human PH20, are translated with a cleavable N-terminal signal peptide that directs the protein to the endoplasmic reticulum (ER). At the C-terminus of these proteins is another signal sequence that directs addition of a preformed GPI-anchor to the polypeptide within the lumen of the ER. Addition of the GPI anchor occurs following cleavage of the C-terminal portion at a specific amino acid position, called the co-sitew-site (typically located approximately 20-30 amino acids from the C-terminus). Although there appears to be no consensus sequence to identify the location of the co-sitew-site, GPI anchored proteins contain a C-terminal GPI-anchor attachment signal sequence or domain that typically contains a predominantly hydrophobic region of 8-20 amino acids, preceded by a hydrophilic spacer region of 8-12 amino acids immediately downstream of the co-sitew-site. This hydrophilic spacer region often is rich in charged amino acids and proline (White et al. (2000) J. Cell Sci. 113(Pt.4):721-727). There is generally a region of approximately 11 amino acids before the .omega.-1ω−1 position that is characterized by a low amount of predicted secondary structure, a region around the cleavage site (.omega.-siteω-site), from .omega.-1ω−1 to .omega.ω+2 that is characterized by the presence of small side chain residues, the spacer region between positions .omega.ω+3 and .omega.ω+9, and a hydrophobic tail from .omega.ω+10 to the C-terminal end (Pierleoni et al., (2008) BMC Bioinformatics 9:392).

[0240](135) Although there is no GPI-anchor attachment signal consensus sequence, various in silico methods and algorithms have been developed that can be used to identify such sequences in polypeptides (see, e.g., Udenfriend et al. (1995) Methods Enzymol. 250:571-582; Eisenhaber et al. (1999) J. Mol. Chem. 292: 741-758; Kronegg and Buloz, (1999), ""Detection/prediction of GPI cleavage site (GPI-anchor) in a protein (DGPI),"" 129.194.185.165/dgpi/; Fankhauser et al. (2005) Bioinformatics 21:1846-1852; Omaetxebarria et al. (2007) Proteomics 7:1951-1960; Pierleoni et al. (2008) BMC Bioinformatics 9:392), including those that are readily available on bioinformatic websites, such as the ExPASy Proteomics tools site (expasy.ch/tools/). Thus, one of skill in the art can determine whether a PH20 polypeptide likely contains a GPI-anchor attachment signal sequence, and, therefore, whether the PH20 polypeptide is a GPI-anchored protein.

[0241](136) The covalent attachment of a GPI-anchor to the C-terminus of human PH20 and, therefore, the membrane-bound nature of PH20, has been confirmed using phosphatidylinositol-specific phospholipase C (PI-PLC) hydrolysis studies (see e.g., Lin et al., (1994) J. Biol. Chem. 125:1157-1163). Phosphatidylinositol-specific phospholipase C (PI-PLC) and D (PI-PLD) hydrolyze the GPI anchor, releasing the PH20 polypeptide from the cell membrane. The prior art literature reports that a .omega.-sitew-site cleavage site of human PH20 is identified between Ser-490 and Ala-491 and for monkey PH20 is identified between Ser491 and Thr492 (Lin et al. (1993) Proc. Natl. Acad. Sci, (1993) 90:10071-10075). Thus, the literature reports that a GPI-anchor attachment signal sequence of human PH20 is located at amino acid positions 491-509 of the precursor polypeptide set forth in SEQ ID NO:6, and the co-sitew-site is amino acid position 490. Thus, in this modeling of human PH20, amino acids 491-509 are cleaved following transport to the ER and a GPI anchor is covalently attached to the serine residue at position 490.

[0242](137) 2. Function

[0243](138) PH20 is normally expressed in sperm from a single testis-specific gene. PH20 is a sperm-associated protein involved in fertilization. PH20 is normally localized on the sperm surface, and in the lysosome-derived acrosome, where it is bound to the inner acrosomal membrane. PH20 is multifunctional and exhibits hyaluronidase activity, hyaluronan (HA)-mediated cell-signaling activity, and acts as a sperm receptor for the zona pellucida surrounding the oocyte when present on acrosome reacted (AR) sperm. For example, PH20 is naturally involved in sperm-egg adhesion and aids penetration by sperm of the layer of cumulus cells by digesting hyaluronic acid. In addition to being a hyaluronidase, PH20 also appears to be a receptor for HA-induced cell signaling, and a receptor for the zona pellucida surrounding the oocyte. Due to the role of PH20 in fertilization, PH20 can be used as an antigen for immunocontraception.

[0244](139) PH20 is a neutral active hyaluronidase, although it can exhibit acid-active activity in some cases. The hyaluronidase activity of PH20 is exhibited by the plasma membrane- and inner acrosomal membrane-associated PH20. The plasma membrane PH20 exhibits hyaluronidase activity only at neutral pH, while the inner acrosomal membrane-associated PH20 exhibits acid-active enzyme activity. The structural basis for these differences is due to the presence of two catalytic sites in PH20. A first catalytic site is designated the Peptide 1 region, corresponding to amino acid residues 142-172 of SEQ ID NO:6, which is involved in enzyme activity of PH20 at neutral pH. A second catalytic site is designated the peptide 3 region, corresponding to amino acid residues 277-297 of SEQ ID NO:6, which is involved in enzyme activity at lower pH. A change in the structure of the inner acrosomal membrane-associated PH20 occurs after the acrosome reaction, whereby PH20 is endoproteolytically cleaved but held together by disulfide bonds. The result of the endoproteolysis is that the peptide 3 region is activated and can thus effect neutral and acid-activity to PH20 (see e.g., Cherr et al. (2001) Matrix Biology, 20:515-525. Also, after the acrosome reaction, lower molecular weight forms are generated by release from the inner acrosomal membrane (e.g., a 53 kDa soluble form of PH20 is generated in monkey). The lower molecular weight form(s) also is acid active.

[0245](140) The hyaluronidase activity of PH20 accounts for the spreading activity observed in animal testes extracts that have been used clinically for decades to increase the dispersion and absorption of drugs (see e.g., Bookbinder et al. (2006) J Controlled Release, 114:230-241). For example, pharmaceutical preparations containing hyaluronidase were developed as fractionated extracts from bovine testes for therapeutic use as spreading agents and in other applications (Schwartzman (1951) J. Pediat., 39:491-502). Original bovine testicular extract preparations included, for example, extracts sold under the trademarks Wydase.RTM.®, Hylase.RTM.®, "“Dessau,"” Neopermease.RTM.®, Alidase.RTM.® and Hyazyme.RTM.®. It is now known that the spreading activity of testicular extract preparations are due to PH20 hyaluronidase activity. For example, in 2001 a sperm hyaluronidase in bull was identified as the hyaluronidase PH20 (Lalancette et al. (2001) Biol. Reprod., 65:628-36). By catalyzing the hydrolysis of hyaluronic acid, PH20 hyaluronidase lowers the viscosity of hyaluronic acid, thereby increasing tissue permeability. Hence, soluble forms of PH20 are used as a spreading or dispersing agent in conjunction with other agents, drug and proteins to enhance their dispersion and delivery, and to improve the pharmacokinetic and pharmacodynamic profile of the coadministered agent, drug or protein (see e.g., U.S. Pat. No. 7,767,429; Bookbinder et al. (2006) J Controlled Release, 114:230-241).

[0246](141) 3. Soluble PH20 Polypeptides

[0247](142) PH20 can exist in membrane-bound or membrane-associated form, or can be secreted into the media when expressed from cells, and thereby can exist in soluble form. Soluble PH20 can be detected and discriminated from insoluble, membrane-bound PH20 using methods well known in the art, including, but not limited to, those using a Triton.RTM.® X-114 assay. In this assay, soluble PH20 hyaluronidases partition into the aqueous phase of a Triton.RTM.® X-114 solution warmed to 37.degree.° C. (Bordier et al., (1981) J. Biol. Chem., 256:1604-7) while membrane-anchored PH20 hyaluronidases partition into the detergent rich phase. Thus, in addition to using algorithms to assess whether a PH20 polypeptide is naturally GPI-anchored and hence membrane-bound, solubility experiments also can be performed.

[0248](143) Soluble PH20 enzymes include hyaluronidases that contain a GPI-anchor attachment signal sequence, but that are loosely attached to the membrane such that they do not contain a phospholipase sensitive anchor. For example, soluble PH20 polypeptides include ovine or bovine PH20. Various forms of such soluble PH20 hyaluronidases have been prepared and approved for therapeutic use in subjects, including humans. For example, animal-derived hyaluronidase preparations include Vitrase.RTM.® (ISTA Pharmaceuticals), a purified ovine testicular hyaluronidase, and Amphadase.RTM.® (Amphastar Pharmaceuticals), a bovine testicular hyaluronidase. Soluble PH20 enzymes also include truncated forms of non-human or human membrane-associated PH20 hyaluronidases that lack one or more amino acid residues of a glycosylphosphatidylinositol (GPI) anchor attachment signal sequence and that retain hyaluronidase activity (see e.g., U.S. Pat. No. 7,767,429; U.S. Publication No. US20100143457). Thus, instead of having a GPI-anchor covalently attached to the C-terminus of the protein in the ER and being anchored to the extracellular leaflet of the plasma membrane, these polypeptides are secreted when expressed from cells and are soluble. In instances where the soluble hyaluronan degrading enzyme retains a portion of the GPI anchor attachment signal sequence, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid residues in the GPI-anchor attachment signal sequence can be retained, provided the polypeptide is soluble (i.e., secreted when expressed from cells) and active.

[0249](144) Exemplary soluble hyaluronidases that are C-terminally truncated and lack all or a portion of the GPI anchor attachment signal sequence include, but are not limited to, PH20 polypeptides of primate origin, such as, for example, human and chimpanzee PH20 polypeptides. For example, soluble PH20 polypeptides can be made by C-terminal truncation of a polypeptide set forth in SEQ ID NOS:7, 10, 12, 14, 69, 72, 857, 859, 861 or 870 or variants thereof that exhibit at least 80%, 85%, 90%, 95% or more sequence identity to any of SEQ ID NO: 7, 10, 12, 14, 69, 72, 857, 859, 861 or 870, wherein the resulting polypeptide is active, soluble and lacks all or a portion of amino acid residues from the GPI-anchor attachment signal sequence.

[0250](145) Exemplary soluble PH20 polypeptides are C-terminal truncated human PH20 polypeptides that are mature (lacking a signal sequence), soluble and exhibit neutral activity, and that contain a contiguous sequence of amino acids set forth in SEQ ID NO:6 or SEQ ID NO:7 that minimally has a C-terminal truncated amino acid residue at or after amino acid residue 464 of the sequence of amino acids set forth in SEQ ID NO:6. For example, soluble PH20 polypeptides include C-terminal truncated polypeptides that minimally contain a contiguous sequence of amino acids 36-464 of SEQ ID NO:6, or includes a sequence of amino acids that has

at least 85%, for example at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity to a contiguous sequence of amino acids that has a C-terminal amino acid residue after amino acid 464 of SEQ ID NO:6 and retains hyaluronidase activity. Exemplary C-terminally truncated human PH20 polypeptides are mature polypeptides (lacking a signal sequence) that include a contiguous sequence of amino acids set forth in SEQ ID NO:6 with a C-terminal residue after 464 such as after amino acid position 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499 or 500 of the sequence of amino acids set forth in SEQ ID NO:6, or a variant thereof that exhibits at least 85% sequence identity, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity thereto and retains hyaluronidase activity. For example, exemplary C-terminal PH20 polypeptides have a sequence of amino acids 36 to 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499 or 500 of the sequence of amino acids set forth in SEQ ID NO:6, or a variant thereof that exhibits at least 85% sequence identity, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity thereto and retains hyaluronidase activity. Soluble PH20 polypeptides include any that has the sequence of amino acids set forth in SEQ ID NOS: 3 or 32-66 or a sequence of amino acids that exhibits at least 85% sequence identity, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66.

~~[0251]~~(146) In particular, a soluble human PH20 polypeptide is a polypeptide that is truncated after amino acid 482 of the sequence set forth in SEQ ID NO:6. Such a polypeptide can be generated from a nucleic acid molecule containing a signal sequence and encoding amino acids 36-482, for example, as set forth in SEQ ID NO:1 (containing an IgG kappa signal sequence) or SEQ ID NO:67 (containing the native signal sequence). Post translational processing removes the signal sequence, leaving a 447 amino acid soluble recombinant human PH20 (SEQ ID NO:3). A product produced upon expression of a vector set forth in SEQ ID NO:4 or 5, and containing a nucleic acid molecule set forth in SEQ ID NO:67, results in a secreted product, designated rHuPH20, in the culture medium that exhibits heterogeneity at the C-terminus such that the product includes a mixture of species that can include any one or more of SEQ ID NOS: 3 and 44-48 in various abundance. Typically, rHuPH20 is produced in cells that facilitate correct N-glycosylation to retain activity, such as mammalian cells, for example CHO cells (e.g., DG44 CHO cells). Hylenex~~.RTM.~~® (Halozyme) is a human recombinant hyaluronidase produced by genetically engineered Chinese Hamster Ovary (CHO) cells containing nucleic acid encoding a truncated human PH20 polypeptide (designated rHuPH20).

C. ~~Modified~~MODIFIED PH20 ~~Polypeptides~~POLYPEPTIDES

~~[0252]~~(147) Provided herein are modified or variant PH20 polypeptides. The modified PH20 polypeptides provided herein exhibit altered activities or properties compared to a wildtype, native or reference PH20 polypeptide. Included among the modified PH20 polypeptides provided herein are PH20 polypeptide that are active mutants, whereby the polypeptides exhibit at least 40% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid modification (e.g., amino acid replacement). In particular, provided herein are PH20 polypeptides that exhibit hyaluronidase activity and that exhibit increased stability compared to

the PH20 not containing the amino acid modification. Also provided are modified PH20 polypeptides that are inactive, and that can be used, for example, as antigens in contraception vaccines.

[0253](148) The modifications can be a single amino acid modification, such as single amino acid replacements (substitutions), insertions or deletions, or multiple amino acid modifications, such as multiple amino acid replacements, insertions or deletions. Exemplary modifications are amino acid replacements, including single or multiple amino acid replacements. The amino acid replacement can be a conservative substitution, such as set forth in Table 2, or a non-conservative substitution, such as any described herein. Modified PH20 polypeptides provided herein can contain at least or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more modified positions compared to the PH20 polypeptide not containing the modification.

[0254](149) The modifications described herein can be in any PH20 polypeptide, including, including precursor, mature, or C-terminal truncated forms, so long as the modified form exhibits hyaluronidase activity. For example, the PH20 polypeptides contain modifications compared to a wildtype, native or reference PH20 polypeptide set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870, or in a polypeptide that has a sequence of amino acids that is at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 6-66, 68-72, 856-861, 869 or 870. For example, the modifications are made in a human PH20 polypeptide having the sequence of amino acids including or set forth in SEQ ID NO:7, SEQ ID NO:69 or SEQ ID NO:72; a bovine PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NOS:16 or 18; a rabbit PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:24; a Cynomolgus monkey PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:14; a guinea pig PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:29; a rat PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:22; a mouse PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:20; a chimpanzee PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:10 or 870; a Rhesus monkey PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:12; a Fox PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:31; a Gibbon PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:857; a Marmoset PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO: 859; an Orangutan PH20 polypeptide having a sequence of amino acids including or set forth in SEQ ID NO:861; or a sheep PH20 polypeptide having a sequence of amino acids including or set forth in any of SEQ ID NOS: 25-27; or in sequence variants or truncated variants that exhibit at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 7, 10, 12, 14, 16, 18, 20, 22, 24-27, 29, 31, 69, 72, 857, 859, 861 or 870.

[0255](150) In particular, provided herein are PH20 polypeptides that contain modifications compared to a PH20 polypeptide set forth in SEQ ID NO: 3, 7, 32-66, 69 or 72, or a polypeptide that has a sequence of amino acids that is at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. For example, the modifications provided herein also can be made in a PH20 polypeptide set forth as SEQ ID NO: 10, 12, 14, 24, 857, 859, 861 or 870.

[0256](151) In particular, provided herein are modified soluble PH20 polypeptides that are PH20 polypeptides containing a modification provided herein, and that when expressed from cells are secreted into the media as a soluble protein. For example, the modifications are made in a soluble PH20 polypeptide that is C-terminally truncated within or near the C-terminus portion containing the GPI-anchor signal sequence of a PH20 polypeptide that contains a GPI-anchor signal sequence. The C-terminal truncation can be a truncation or deletion of 8 contiguous amino acids at the C-terminus, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more amino acids at the C-terminus, so long as the resulting C-terminally truncated polypeptide exhibits hyaluronidase activity and is secreted from cells (e.g., into the media) when expressed. In some examples, the modifications provided herein are made in a soluble PH20 polypeptide that is a C-terminally truncated polypeptide of SEQ ID NO:7, 10, 12, 14, 69, 72, 857, 859, 861 or 870 or a variant thereof that exhibits at least 70%, 75%, 80%, 85%, 86%), 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 7, 10, 12, 14, 69, 72, 857, 859, 861 or 870. In particular, the modifications provided herein are made in a soluble or C-terminally truncated human PH20 polypeptide having the sequence of amino acids set forth in SEQ ID NOS: 3 or 32-66 or a sequence of amino acids that exhibits at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66. For example, modified PH20 polypeptides provided herein contain amino acid replacements or substitutions, additions or deletions, truncations or combinations thereof with reference to the PH20 polypeptide set forth in SEQ ID NO:3.

[0257](152) Modifications also can be made in the corresponding precursor form containing a signal peptide of any of SEQ ID NOS: 3, 7, 10, 12, 14, 16, 18, 20, 22, 24-27, 29,31,32-6629, 31, 32-66, 69, 72, 857, 859, 861 or 870. For example, modifications provided herein can be made in a precursor form set forth in any of SEQ ID NOS: 2, 6, 8, 9, 11, 13, 15, 17, 19, 21, 23, 28, 30, 856, 858, 860 or 869 or in a variant thereof that exhibits at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 2, 6, 8, 9, 11, 13, 15, 17, 19, 21, 23, 28, 30, 856, 858, 860 or 869.

[0258](153) In examples of modified PH20 polypeptides provided herein, the modified PH20 polypeptide does not contain the sequence of amino acids set forth in any of SEQ ID NOS: 3-66, 68-72, 856-861, 869 or 870. Typically, the modified PH20 polypeptide is a human PH20 polypeptide, and does not contain the sequence of amino acids set forth in any of SEQ ID NOS: 8-31, 856-861, 869 or 870.

[0259](154) Generally, any modification, such as amino acid replacement, deletion or substitution, can be made in a PH20 polypeptide, with the proviso that the modification is not an amino acid replacement where the only modification is a single amino acid replacement that is V12A, N47A, D111N, E113Q, N131A, R176G, N200A, N219A, E249Q, R252T, N333A or N358A. Also, where the modified PH20 polypeptide contains only two amino acid replacements, the amino acid replacements are not P13A/L464W, N47A/N131A, N47A/N219A, N131A/N219A or N333A/N358A. In a further example, where the modified PH20 polypeptide contains only three amino acid replacements, the amino acid replacements are not N47A/N131A/N219A. Exemplary modifications provided herein are described in detail below.

[0260](155) For purposes herein, reference to positions and amino acids for modification herein, including amino acid replacement or replacements, are with reference to the PH20 polypeptide set forth in SEQ ID NO:3. It is within the level of one of skill in the art to make any of the modifications provided herein in another PH20 polypeptide by identifying the corresponding amino acid residue in another PH20 polypeptide, such as any set forth in SEQ ID NOS: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24-27, 28, 29, 30, 31, 32-66, 68-72, 856, 857, 858, 859, 860, 861, 869 or 870 or a variant thereof that exhibits at least 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24-27, 28, 29, 30, 31, 32-66, 68-72, 856, 857, 858, 859, 860, 861, 869 or 870. Corresponding positions in another PH20 polypeptide can be identified by alignment of the PH20 polypeptide with the reference to the PH20 polypeptide set forth in SEQ ID NO:3. For example, FIG. 2 (A-L) depicts alignment of exemplary PH20 polypeptides with SEQ ID NO:3, and identification of exemplary corresponding positions. Also, since SEQ ID NOS: 3, 7, 32-66, 69 and 72 are all forms of a mature human PH20 with a different C-terminal amino acid residue, the numbering of amino acid residues in any of SEQ ID NOS: 7, 32-66, 69 and 72 is the same as SEQ ID NO:3, and hence the corresponding residues of each are identical to that set forth in SEQ ID NO:3 (see e.g., FIG. 1). Further, SEQ ID NOS set forth in any of SEQ ID NOS: 2, 6, 70 or 71 are precursor forms thereof that differ by only the presence of a signal sequence. For purposes of modification (e.g., amino acid replacement), the corresponding amino acid residue can be any amino acid residue, and need not be identical to the residue set forth in SEQ ID NO:3. Typically, the corresponding amino acid residue identified by alignment with residues in SEQ ID NO:3 is an amino acid residue that is identical to SEQ ID NO:3, or is a conservative or semi-conservative amino acid residue thereto (see e.g., FIGS. 2A-2L). It is also understood that the exemplary replacements provided herein can be made at the corresponding residue in a PH20 polypeptide, so long as the replacement is different than exists in the unmodified form of the PH20 polypeptide. Based on this description and the description elsewhere herein, it is within the level of one of skill in the art to generate a modified PH20 polypeptide containing any one or more of the described mutation, and test each for a property or activity as described herein.

[0261](156) Modifications in a PH20 polypeptide also can be made to a PH20 polypeptide that also contains other modifications, including modifications of the primary sequence and modifications not in the primary sequence of the polypeptide. For example, modifications described herein can be in a PH20 polypeptide that is a fusion polypeptide or chimeric polypeptide. The modified PH20 polypeptides provided herein also include polypeptides that are conjugated to a polymer, such as a PEG reagent.

[0262](157) Also provided herein are nucleic acid molecules that encode any of the modified PH20 polypeptides provided herein. In particular examples, the nucleic acid sequence can be codon optimized, for example, to increase expression levels of the encoded sequence. The particular codon usage is dependent on the host organism in which the modified polypeptide is expressed. One of skill in the art is familiar with optimal codons for expression in mammalian or human cells, bacteria or yeast, including for example E. coli or Saccharomyces cerevisiae. For example, codon usage information is available from the Codon Usage Database available at kazusa.or.jp.codon (see Richmond (2000) Genome Biology, 1:reports241 for a description of the database). See also, Forsburg (1994) Yeast, 10:1045-1047; Brown et al. (1991) Nucleic Acids Research, 19:4298; Sharp et al. (1988) Nucleic Acids Res., 12:8207-8211; Sharp et al. (1991)

Yeast, 657-78). In some examples, the encoding nucleic acid molecules also can be modified to contain a heterologous signal sequence to alter (e.g., increased) expression and secretion of the polypeptide. Exemplary of a heterologous signal sequence is a nucleic acid encoding the IgG kappa signal sequence (set forth in SEQ ID NO:868).

~~[0263]~~(158) The modified polypeptides and encoding nucleic acid molecules provided herein can be produced by standard recombinant DNA techniques known to one of skill in the art. Any method known in the art to effect mutation of any one or more amino acids in a target protein can be employed. Methods include standard site-directed or random mutagenesis of encoding nucleic acid molecules, or solid phase polypeptide synthesis methods. For example, nucleic acid molecules encoding a PH20 polypeptide can be subjected to mutagenesis, such as random mutagenesis of the encoding nucleic acid, error-prone PCR, site-directed mutagenesis, overlap PCR, gene shuffling, or other recombinant methods. The nucleic acid encoding the polypeptides can then be introduced into a host cell to be expressed heterologously. Hence, also provided herein are nucleic acid molecules encoding any of the modified polypeptides provided herein. In some examples, the modified PH20 polypeptides are produced synthetically, such as using solid phase or solutions phase peptide synthesis.

~~[0264]~~(159) In the subsections below, exemplary modified PH20 polypeptide exhibiting altered properties and activities, and encoding nucleic acid molecules, provided herein are described.

~~[0265]~~(160) 1. Active Mutants

~~[0266]~~(161) Provided herein are modified PH20 polypeptides that contain one or more amino acid replacements in a PH20 polypeptide and that exhibit hyaluronidase activity. The modified PH20 polypeptides can exhibit 40% to 5000% of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the polypeptide set forth in SEQ ID NOS: 3 or 7. For example, modified PH20 polypeptides provided herein exhibit at least 40% of the hyaluronidase activity, such as at least 50%, 60%, 70%, 80%, 90%, 100%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%~~)~~, 2000%, 3000% or more of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the corresponding polypeptide not containing the amino acid modification (e.g., amino acid replacement), for example, a polypeptide set forth in SEQ ID NO:3 or 7. For example, exemplary positions that can be modified, for example by amino acid replacement or substitution, include, but are not limited to, any of positions corresponding to position 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 20, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 58, 59, 60, 61, 63, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 77, 79, 81, 82, 83, 84, 85, 86, 87, 89, 90, 91, 92, 93, 94, 96, 97, 98, 99, 102, 103, 104, 105, 106, 107, 108, 110, 114, 117, 118, 119, 120, 122, 124, 125, 127, 128, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 186, 192, 193, 195, 196, 197, 198, 200, 202, 204, 205, 206, 208, 209, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 224, 226, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 242, 245, 247, 248, 251, 253, 255, 256, 257, 258, 259, 260, 261, 263, 264, 265, 266, 267, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 297, 298, 300, 301, 302, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315,

316, 317, 318, 320, 321, 323, 324, 325, 326, 327, 328, 331, 334, 335, 338, 339, 342, 343, 347, 348, 349, 351, 353, 356, 357, 358, 359, 360, 361, 367, 368, 369, 371, 373, 374, 375, 376, 377, 378, 379, 380, 381, 383, 385, 387, 388, 389, 391, 392, 393, 394, 395, 396, 397, 398, 399, 401, 403, 404, 405, 406, 407, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 425, 426, 427, 428, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. Typically, the amino acid residue that is modified (e.g., replaced with another amino acid) at the position corresponding to any of the above positions in a PH20 polypeptide is an identical residue, a conservative residue or a semi-conservative amino acid residue to the amino acid residue set forth in SEQ ID NO:3.

~~[0267]~~(162) To retain hyaluronidase activity, modifications typically are not made at those positions that are less tolerant to change or required for hyaluronidase activity. For example, generally modifications are not made at a position corresponding to position 7, 16, 17, 18, 19, 21, 25, 53, 55, 56, 57, 62, 64, 76, 78, 80, 88, 95, 100, 101, 109, 111, 112, 113, 115, 116, 121, 123, 126, 129, 185, 187, 188, 189, 190, 191, 194, 199, 201, 203, 207, 210, 223, 225, 227, 228, 229, 241, 243, 244, 246, 249, 250, 252, 254, 262, 268, ~~282,~~ 295, 296, ~~298,~~ 299, 303, 319, 322, 329, 330, 332, 333, 336, 337, 340, 341, 344, 345, 346, 350, 352, 354, 355, 362, 363, 364, 365, 366, 370, 372, 382, 384, 386, 390, 400, 402, 408, 423, 424, 429, 430~~, 431~~, with reference to amino acid positions set forth in SEQ ID NO:3. Also, in examples where modifications are made at any of positions 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 20, 22, 23, 27, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 58, 59, 60, 61, 63, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 77, 79, 81, 82, 83, 84, 85, 86, 87, 89, 90, 91, 92, 94, 96, 98, 99, 102, 103, 104, 105, 106, 107, 108, 110, 114, 117, 118, 119, 122, 124, 125, 127, 128, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 143, 144, 145, 149, 150, 152, 153, 154, 155, 156, 157, 158, 159, 161, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 186, 192, 193, 195, 197, 198, 200, 202, 204, 206, 208, 209, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 224, 226, 230, 231, 232, 233, 234, 235, 236, 238, 239, 240, 242, 245, 247, 248, 251, 253, 255, 256, 257, 258, 260, 261, 263, 264, 265, 266, 267, 269, 270, 271, 272, 273, 274, 275, 276, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 297, 298, 300, 301, 302, 304, 305, 306, 307, 308, 310, 311, 312, 313, 314, 315, 316, 317, 318, 320, 321, 323, 324, 325, 326, 327, 331, 334, 335, 338, 339, 342, 343, 347, 348, 349, 351, 353, 356, 357, 358, 359, 360, 361, 367, 368, 369, 371, 373, 374, 375, 376, 377, 378, 379, 380, 381, 383, 385, 387, 388, 389, 391, 392, 393, 394, 395, 396, 397, 398, 399, 401, 403, 404, 405, 406, 410, 411, 412, 413, 414, 415, 416, 417, 419, 420, 422, 425, 426, 427, 428, 431, 432, 434, 437, 438, 439, 440, 441, 442, 443, 444, or 447 with reference to amino acid positions set forth in SEQ ID NO:3, the modification(s) is/are not the corresponding amino acid ~~replacements~~replacement(s) set forth in Table 5 or 10 herein, which are amino acid replacements that result in an inactive polypeptide. For example, if the modification is a modification at a position corresponding to position 2 with reference to SEQ ID NO:3, the modification is not replacement to a histidine (H), lysine (K), tryptophan (W) or tyrosine (Y).

~~[0268]~~(163) Exemplary amino acid replacements at any of the above corresponding positions are set forth in Table 3. Reference to the corresponding amino acid position in Table 3 is with reference to positions set forth in SEQ ID NO:3. It is understood that the replacements can be made in the corresponding position in another PH20 polypeptide by alignment therewith with the sequence set forth in SEQ ID NO:3 (see e.g., FIGS. 1 and 2), whereby the corresponding

position is the aligned position. In particular examples, the amino acid replacement(s) can be at the corresponding position in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto, so long as the resulting modified PH20 polypeptide exhibits at least 40% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid replacement. In particular, the ~~replacements~~replacement(s) can be in a corresponding position in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a variant thereof that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%~~)~~, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. In one example, any one or more of the replacements are in SEQ ID NO:3, so long as the resulting modified PH20 polypeptide exhibits at least 40% of the hyaluronidase activity of the PH20 polypeptide set forth in SEQ ID NO:3.

(164) TABLE-US-00003 TABLE 3 Active Mutants ~~Corres- Corres- Corres- ponding ponding ponding Posi- Posi- Posi- tion~~Corre- Corre- Corre- sponding sponding sponding Position Replacement ~~tion~~Position Replacement ~~tion~~Position Replacement 1 A C E F G ~~H K N P~~ 2 A C G I L ~~P Q S T~~ 3 E H L Y H K N P Q P Q S T V R S T V W ~~V~~ 4 A I S T V 5 H 6 A H K L N Q R 7 M 8 I L M P 9 K L Q R S V 10 D E G H N ~~Q R S W~~ 11 D G H K S 12 A E I K L Q R S W N R S T 13 H S T Y 14 D I M V 15 A M V 20 S 22 H M T Y 23 D 24 A E G H I ~~K L M N~~ 26 A E G H I ~~K M P Q~~ 27 A D E F H K L M N R K M P Q R I K L P ~~R~~Q T V Y ~~R~~S T V W Y ~~Q~~R S T W 28 A D E F I ~~L M N P~~ 29 A E G H I ~~K L M P~~ 30 A F G H K L M N P R ~~S T V W~~K L M P R ~~S T V W~~L M P Q R S T V W S T V W S T V W 31 A C G H I ~~K L P R S~~ 32 A C F G H ~~K L M N~~ 33 G M P Q R K L P R S K L M N Q S T W T V W Y ~~Q~~R S T V W Y 34 A E H K Q ~~R W~~ 35 F H L Q T ~~V Y~~ 36 A D G H K R W V Y L N R T 37 F I K M P ~~R W V~~ 38 Y 39 A L N Q R R W V T Y 40 L W 41 A C D E G 42 A H N T V ~~42 A~~ W 43 N T 44 E 45 I K 46 A C E F H ~~L M N R~~ 47 A D F G H ~~K M Q~~ 48 F G H I K L M N R S K M Q R S M N Q R S T V Y ~~R S~~ T W Y ~~S~~ V Y 49 I K R S V 50 A C D E H ~~L M Q R~~ 51 A N R S L M Q R S V Y 52 N P Q R S ~~T~~ 54 A F N Q S ~~V~~ 58 C G H I K T V L N P Q R S W Y 59 Q N 60 K 61 F I M V 63 A H I K L ~~M N R S~~ 65 R 66 H R M N R S T V W 67 F L R V Y 68 E G H K L ~~P Q R S~~ 69 A C E F G P Q R S T I L M P ~~T~~ R T W Y 70 A C F G H ~~K L N P~~ 71 A D G H L ~~M N Q~~ 72 A D E H K K L N P R M N Q R S L M Q R S S T V Y ~~R S R S~~ Y 73 A C D G H ~~K L M Q~~ 74 A C E F G ~~H K L M~~ 75 A C F H L K L M Q R H K L M N M N Q R S S T W ~~N~~ P R S V W ~~R S~~ T Y 77 H K 79 L T V 81 P 82 A E G H I L M N Q R S T V 83 F G H K L ~~N Q R S~~ 84 D E F G H 85 V N Q R S T I L M N ~~85~~P V ~~T V P~~ Q R T W Y 86 A D E F G ~~H I K L~~ 87 A C E G H ~~I L M P~~ 89 C K M P R ~~W~~H I K L M I L M P Q W N P R S T ~~V W Q~~ R S T V Y V W 90 A E G H I ~~K L N Q~~ 91 A Q R 92 C H L M T ~~V~~K L N Q R V S T W 93 D E F G H ~~I L M N~~ 94 A C D E F ~~H L M N~~ 96 D L V I L M N P H L M N Q Q R S T V ~~Q~~ R S T 97 A C D E F ~~G I L N P~~ 98 A C D E H ~~I L M Q~~ 99 A R S G I L

N P I L M Q R Q R S W Y R S V W 102 A C E G H K L M N Q R S T W 103 N 104 A C G I K M R S T 105 A C G H I M R S T P Q R S T W V 106 V W V 107 F I L 108 G 110 V 114 A G H M S 117 D 118 H K L M N Q V 119 F P Q Y 120 D F G H I 122 M L N P R 122 M S T V W Y 124 H L R 125 A H R S 127 A E G H L M N Q R S T V W 128 A C G I K L Q R S 130 I R 131 C E F G H L Q R S W I L M Q W R S T V Y 132 A C E F H I K L N 133 I 134 L T V I K L N Q S T V Y 135 A C D F G H K L N 136 A C D F H I M N Q 137 A C I T A H K L N Q I M N Q R C H I L Q M R S W Y R S T W M N R S W Y 139 A C D E F G H K L 140 A C D F G H I K L 141 A D E F G G H K L M H I K L M H L M M Q R R S T V M R V W Y Q R S T V W Y 142 C D E G H I K L M 143 C E G I K L N V 144 R T W I K L M N L N V P Q R S T 145 A C D E G H L M N 146 A C E G H I K N P 147 A C D F G H L M N P I K N P Q I L M P P Q R Q R S T V Y Q R S V W Y 148 C F G H I K L Q R S 149 C G K L M Q R S T 150 A C D E F K L Q R S Q R S T V G I L N P T V W Y V P R S W Y 151 A C G H K L M N Q 152 A C F I M R T V W 153 I L S L M N Q R S R T V W Y S T V W Y 154 I R T V 155 A C D F G H K L M 156 A C D G I H K L M R L M Q R R S S T V W S T V W 157 W 158 A F G H L Q S 159 A D E G H Q S L M N Q R S V 160 C F G H I K L M N 161 A C D E R S V 162 A D E G H K L M N Q S V L M P Q R R S W V Y Q R S V W Y 163 A E G K L Q R S T 164 L M V W 165 A C D F N Q R S T V R S V V W W Y W 166 A C E F G H L N Q 167 A D G H K 168 H H L N Q R M N P 168 H R S T W Y R S T Y 169 L R V 170 A Q N R V 171 I V 172 A C 173 Q N R 174 A G H K M N Q R S T V W Y 175 E H T V Y 176 K L 177 V 178 G K M R 179 A C E G I K L M N 180 F G I K M 181 K M Q K L M N P R S T V 182 L 183 E L 184 W 186 Y 192 S T 193 F G Q R S Y 195 A G H I L Y N Q R S T W V 196 E G L N R S T W Y 197 A D E F G H K L M 198 A D E H L S T W Y H K L M Q N Q R S Q T R S T W T W Y 200 D T 202 M 204 P W 205 L R S T V W Y 206 H I K L M Q R S T 208 A C K L M W Y Q R S T Q R S T V 209 A E F G L 211 L W N R S T 211 L W 212 N S T 213 A E G H K L M N Q R V W Y 214 Q 215 A D E G H 217 M K L M 217 M Q R T V W Y 218 F M V 219 A C D E H I K L M 220 A D H I L I K L M R M S T V R S T W 221 A C I M Q T V 222 D F G I K 224 I T V L N R S 224 I V 226 W 230 I 231 T 232 S 233 A F G K L R Y 234 L M 235 A E G H K R Y T 236 A G H K R S 237 A C E F H L N Q R 238 D E H K Q S L N Q R S R S T S T W 239 N 240 K A M P Q R S V 242 F 245 H R S V 247 I L M 248 A H W Y 251 L M Y 253 I 255 A G N Q R S 256 A H L V S 257 A C G I K L M N Q 258 G H N R S 259 E G I K L L M N Q R N P Q R R S T V S T V W Y 260 A D E G H L M Q R 261 A F K M N Q R T V 263 A H K M R L M Q R S Q R T V S Y W T V Y 264 A H 265 I 266 Y 267 M T 269 A C D S 270 M N S T 271 F G L M S V 272 D M R S T 273 H T Y V 274 A F S 275 L V 276 C D E G H I L M R 277 A C D E G I L M R S H K M S Y N

Q Y R S T Y 278 A E F G H I K N R 279 A H Q R T 280 G Q I K N R S T V Y 282 D G M Q 283 E P R S T 284 A E G H L M N Q S T Y 285 A F G H M N Q Y 286 R S W 287 I N T N Q Y 288 L W 289 K S 290 I M 291 C Q R S V 292 A C F G H K N P R 293 A C D F G K N P R V K L M V W P Q 294 M W S V Y 294 M 297 A 298 G I 300 R 301 A V 302 I W 303 D V 304 G I 305 D E N 306 D E S 307 G K N Q S T V W Y 308 D G H K N P R T 309 D E G H K T V W Y P R T L M N Q R S T V W 310 A F G Q R S V Y 311 G H K Q S T 312 G K L N T S V Y T 313 A E G H K L P R S 314 A D H I N Q R S T 315 A E G H K L P R S T Q R S T Y L M R T Y V Y Y T Y 316 D 317 A D H I K M N Q R 318 D F G H I M N Q R S K M N Q S R T W R S T 320 E G H I K L M N R 321 A D H K R S T Y 323 F I L L M N R S S T Y W V Y

324 A D H M N R S 325 A D E G H K M N 326 C K L V Y R S K M N Q S V W 327 M 328 A C G H I K L Q R 331 C E V K L Q R S T V W Y 334 P T 335 S 338 Q 339 M 342 A 343 T V 347 A E G L M R S 348 D G S 349 A E K M N R S R T 351 A C I Q S 353 T V 356 A D H S 357 A C K S T 358 C G L T 359 D E H K M T V 360 T T V 361 H 367 A C G K R S 368 A E G H K S L M R S T V H R S 371 E F G H I K L M R 373 A E F K L M R S V 374 A H I M N K L M R S M R S V P R S T S V V W Y 375 A G I K L M N R S 376 A D E L M Q R S T 377 D E H K P M N R S T Q R S T V R S T Y 378 K N R 379 G H R S T 380 I L P T V W Y 381 E H K N Q R S V 383 A E H I K L M N S 385 A G H N Q R S V L M N S T R S T V V 387 S 388 F H I M R T V W Y 389 A G H K L T V W Y M P Q R S T Y 391 C 392 A F G K L M Q R S 393 A D F H K M Q R S T L M N T R S V W Y R S T 394 L W 395 A G H K R T W 396 A D H L Q T W R S T 397 R 398 L 399 A C E K M N Q R S 401 A E G Q N 403 F N Q R S T V W 404 A P T 405 A F G K M P Q R S 406 A C E F G P Q R S W I N Q S W T Y T V Y 407 A D E F G H L M N 409 A D E G H I P Q R 410 D K M N P Q R S H L M N P I P Q R V W S Q R S T V Q R V W T V Y 411 A H N P R S T V 412 D G H I L N Q P R 413 A E H K N S T V N Q P R S Q R S S T V W Y T 414 I K L M 415 G S W V Y 416 F G H I K L N Q R T V Y 417 I 418 A E F G I L M N P 419 E F G H I L M N P Q K L N R Q S R S V Y S W Y 420 I P 421 A E G H I 422 I T K L M N 422 I T Q R S T Y 425 G I K M N R S Y 426 E G K N P Q S Y 427 H I K Q S R S Y Q S Y T 428 L M P T 431 A E G H I K L N Q 432 E G H N S V K L N Q R V S V W Y 433 A C D E G H I K L 434 F G I M V 435 A C E G H R S T V H I K L P R S T V W Y R S T V W 436 C D E G H I K L M 437 A D G H I K L M Q 438 A C D E G I K L M Q K L M Q R L N P Q Q R R S T W Y R S Y R S T V W 439 A C F G H K L P Q 440 A D E F G H I L M 441 A D F G H K L N S T V W P R Q S V Y H I L M P K L N Q S T V W R S V Y T V Y 442 C G H K L P Q R T 443 A E F G H L M N Q 444 D E F G H P Q R T V L M N Q R I K M N V R W Y R S T W R V W Y 445 A G H L M N P Q R 446 A C D E G H

~~I K L~~ 447 D E F G I N P Q R S H I K L M L M N P ~~S~~Q T V W Y ~~M~~ Q R T V W ~~Q~~ R T V W

~~[0269]~~(165) In particular examples, provided herein is a modified PH20 polypeptide containing an amino acid replacement or replacements at a position or positions corresponding to 1, 6, 8, 9, 10, 11, 12, 14, 15, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 46, 47, 48, 49, 50, 52, 58, 59, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 79, 82, 83, 84, 86, 87, 89, 90, 92, 93, 94, 97, 102, 104, 107, 114, 118, 120, 127, 128, 130, 131, 132, 135, 138, 139, 140, 141, 142, 143, 144, 146, 147, 148, 149, 150, 151, 152, 155, 156, 158, 160, 162, 163, 164, 165, 166, 167, 169, 170, 172, 173, 174, 175, 178, 179, 193, 195, 196, 198, 204, 205, 206, 209, 212, 213, 215, 219, 220, 221, 222, 232, 233, 234, 235, 236, 237, 238, 240, 247, 248, 249, 257, 258, 259, 260, 261, 263, 267, 269, 271, 272, 273, 274, 276, 277, 278, 279, 282, 283, 285, 287, 289, 291, 292, 293, 298, 305, 307, 308, 309, 310, 313, 314, 315, 317, 318, 320, 321, 324, 325, 326, 328, 335, 347, 349, 351, 353, 356, 359, 367, 368, 369, 371, 373, 374, 375, 376, 377, 380, 381, 383, 385, 389, 392, 393, 395, 396, 399, 401, 404, 405, 406, 407, 409, 410, 412, 416, 418, 419, 421, 425, 427, 428, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at positions corresponding to replacement of Leucine (L) at position 1 (L1), P6, V8, I9, P10, N11, V12, F14, L15, A20, S22, F24, L26, G27, K28, F29, D30, E31, P32, L33, D34, M35, S36, L37, F38, S39, F40, I41, I46, N47, A48, T49, G50, G52, V58, D59, Y63, I67, D68, S69, I70, T71, G72, V73, T74, V75, ~~I79~~179, K82, I83, S84, G86, D87, L89, D90, A92, K93, K94, T97, V102, N104, M107, E114, T118, A120, D127, V128, K130, N131, R132, E135, Q138, Q139, Q140, N141, V142, Q143, L144, L146, T147, E148, A149, T150, E151, K152, Q155, E156, E158, A160, K162, D163, F164, L165, V166, E167, I169, K170, G172, K173, L174, L175, N178, H179, H193, K195, K196, G198, F204, N205, V206, K209, D212, D213, S215, N219, E220, S221, T222, T232, Q233, Q234, ~~S235~~5235, P236, V237, A238, T240, V247, R248, E249, P257, D258, A259, K260, S261, L263, A267, T269, ~~1271~~I271, V272, F273, T274, Q276, V277, L278, K279, S282, Q283, E285, V287, T289, G291, E292, T293, A298, G305, L307, S308, I309, M310, M313, K314, S315, L317, L318, D320, N321, E324, T325, I326, N328, T335, Q347, Q349, V351, I353, N356, S359, P367, D368, N369, A371, Q373, L374, E375, K376, G377, F380, T381, R383, K385, E389, E392, Q393, S395, E396, Y399, S401, S404, T405, L406, S407, K409, E410, A412, D416, D418, A419, D421, A425, G427, A428, D431, F433, P436, P437, M438, E439, T440, E441, E442, P443, Q444, I445, F446 or Y447 with reference to amino acid positions set forth in SEQ ID NO:3.

(166) Exemplary amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited, replacement with: histidine (H) at a position corresponding to position 1; A at a position corresponding to position 1; E at a position corresponding to position 1; G at a position corresponding to position 1; K at a position corresponding to position 1; Q at a position corresponding to position 1; R at a position corresponding to position 1; A at a position corresponding to position 6; M at a position corresponding to position 8; Q at a position corresponding to position 9; G at a position corresponding to position 10; H at a position corresponding to position 10; S at a position corresponding to position 11; E at a position corresponding to position 12; I at a position corresponding to position 12; K at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 14; V at a position corresponding to position 15; M at a position corresponding to position 15; S at a position corresponding to position 20; T at a position

corresponding to position 22; E at a position corresponding to position 24; H at a position corresponding to position 24; R at a position corresponding to position 24; A at a position corresponding to position 26; E at a position corresponding to position 26; K at a position corresponding to position 26; M at a position corresponding to position 26; Q at a position corresponding to position 26; R at a position corresponding to position 26; D at a position corresponding to position 27; K at a position corresponding to position 27; R at a position corresponding to position 27; R at a position corresponding to position 28; E at a position corresponding to position 29; I at a position corresponding to position 29; K at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; R at a position corresponding to position 29; S at a position corresponding to position 29; T at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; L at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; G at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; W at a position corresponding to position 31; C at a position corresponding to position 32; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; G at a position corresponding to position 33; W at a position corresponding to position 34; Q at a position corresponding to position 35; V at a position corresponding to position 35; H at a position corresponding to position 36; N at a position corresponding to position 36; F at a position corresponding to position 37; M at a position corresponding to position 37; Y at a position corresponding to position 38; A at a position corresponding to position 39; L at a position corresponding to position 39; N at a position corresponding to position 39; T at a position corresponding to position 39; L at a position corresponding to position 40; T at a position corresponding to position 41; L at a position corresponding to position 46; R at a position corresponding to position 46; D at a position corresponding to position 47; F at a position corresponding to position 47; T at a position corresponding to position 47; W at a position corresponding to position 47, with F at a position corresponding to position 48; H at a position corresponding to position 48; K at a position corresponding to position 48; N at a position corresponding to position 48; R at a position corresponding to position 49; D at a position corresponding to position 50; S at a position corresponding to position 50; M at a position corresponding to position 50; N at a position corresponding to position 52; Q at a position corresponding to position 52; R at a position corresponding to position 52; S at a position corresponding to position 52; T at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; L at a position corresponding to position 58; P at a position corresponding to position 58; Q at a position corresponding to position 58; R at a position corresponding to position 58; H at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; N at a position

corresponding to position 59; K at a position corresponding to position 63; L at a position corresponding to position 63; M at a position corresponding to position 63; R at a position corresponding to position 63; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; P at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; E at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; T at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; Y at a position corresponding to position 70; G at a position corresponding to position 71; N at a position corresponding to position 71; R at a position corresponding to position 71; S at a position corresponding to position 71; K at a position corresponding to position 72; M at a position corresponding to position 72; Q at a position corresponding to position 72; A at a position corresponding to position 73; H at a position corresponding to position 73; K at a position corresponding to position 73; L at a position corresponding to position 73; Q at a position corresponding to position 73; R at a position corresponding to position 73; T at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position corresponding to position 74; E at a position corresponding to position 74; F at a position corresponding to position 74; G at a position corresponding to position 74; H at a position corresponding to position 74; K at a position corresponding to position 74; L at a position corresponding to position 74; M at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; M at position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; L at a position corresponding to position 79; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; E at a position corresponding to position 84; F at a position corresponding to position 84; G at a position corresponding to position 84; N at a position corresponding to position 84; R at a position corresponding to position 84; A at a position corresponding to position 86; H at a position corresponding to position 86; K at a position corresponding to position 86; N at a position corresponding to position 86; S at a position corresponding to position 86; T at a position corresponding to position 86; W at a position corresponding to position 86; C at a position corresponding to position 87; G at a position

corresponding to position 87; L at a position corresponding to position 87; M at a position corresponding to position 87; R at a position corresponding to position 87; S at a position corresponding to position 87; T at a position corresponding to position 87; V at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 89; A at a position corresponding to position 90; E at a position corresponding to position 90; H at a position corresponding to position 90; K at a position corresponding to position 90; N at a position corresponding to position 90; R at a position corresponding to position 90; C at a position corresponding to position 92; L at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; Q at a position corresponding to position 93; R at a position corresponding to position 93; S at a position corresponding to position 93; T at a position corresponding to position 93; D at a position corresponding to position 94; Q at a position corresponding to position 94; R at a position corresponding to position 94; A at a position corresponding to position 97; C at an amino acid residue corresponding to position 97; D at a position corresponding to position 97; E at a position corresponding to position 97; G at a position corresponding to position 97; L at a position corresponding to position 97; S at a position corresponding to position 97; S at a position corresponding to position 102; T at a position corresponding to position 102; R at a position corresponding to position 104; L at a position corresponding to position 107; A at a position corresponding to position 114; Q at a position corresponding to position 118; H at a position corresponding to position 120; F at a position corresponding to position 120; I at a position corresponding to position 120; S at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; E at a position corresponding to position 127; H at a position corresponding to position 127; N at a position corresponding to position 127; Q at a position corresponding to position 127; R at a position corresponding to position 127; I at a position corresponding to position 128; R at a position corresponding to position 130; G at a position corresponding to position 131; I at a position corresponding to position 131; M at a position corresponding to position 131; Q at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; N at a position corresponding to position 132; L at a position corresponding to position 132; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with L at a position corresponding to position 138; T at a position corresponding to position 139; K at a position corresponding to position 140; H at a position corresponding to position 141; R at a position corresponding to position 141; S at a position corresponding to position 141; W at a position corresponding to position 141; Y at a position corresponding to position 141; D at a position corresponding to position 142; G at a position corresponding to position 142; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; Q at a position corresponding to position 142; R at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; R at a position corresponding to position 144; T at a position corresponding to position 144; P at a position corresponding to position 146; R at a position corresponding to position 146; A at a position corresponding to position 147; F at a position corresponding to position 147; L at a position corresponding to position 147; R at a position corresponding to position 147; S at a position corresponding to position 147; V at a position corresponding to position 147; H at a

position corresponding to position 148; K at a position corresponding to position 148; Q at a position corresponding to position 148; T at a position corresponding to position 149; V at a position corresponding to position 149; A at a position corresponding to position 150; D at a position corresponding to position 150; G at a position corresponding to position 150; N at a position corresponding to position 150; S at a position corresponding to position 150; W at a position corresponding to position 150; Y at a position corresponding to position 150; A at a position corresponding to position 151; H at a position corresponding to position 151; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; S at a position corresponding to position 151; T at a position corresponding to position 151; V at a position corresponding to position 151; W at a position corresponding to position 151; Y at a position corresponding to position 151; R at a position corresponding to position 152; T at a position corresponding to position 152; W at a position corresponding to position 152; D at a position corresponding to position 155; G at a position corresponding to position 155; K at a position corresponding to position 155; R at a position corresponding to position 155; D at a position corresponding to position 156; Q at a position corresponding to position 158; S at a position corresponding to position 158; S at a position corresponding to position 160; E at a position corresponding to position 162; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; Q at a position corresponding to position 163; R at a position corresponding to position 163; S at a position corresponding to position 163; M at a position corresponding to position 164; V at a position corresponding to position 164; D at a position corresponding to position 165; F at a position corresponding to position 165; N at a position corresponding to position 165; S at a position corresponding to position 165; V at a position corresponding to position 165; A at a position corresponding to position 166; E at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; W at a position corresponding to position 166; Y at a position corresponding to position 166; D at a position corresponding to position 167; L at a position corresponding to position 169; R at a position corresponding to position 170; A at a position corresponding to position 172; R at a position corresponding to position 173; G at a position corresponding to position 174; K at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 174; T at a position corresponding to position 174; T at a position corresponding to position 175; K at a position corresponding to position 178; R at a position corresponding to position 178; K at a position corresponding to position 179; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; with E at a

position corresponding to position 196; R at a position corresponding to position 196; with D at a position corresponding to position 198; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; L at a position corresponding to position 205; T at a position corresponding to position 205; I at a position corresponding to position 206; K at a position corresponding to position 206; L at a position corresponding to position 206; R at a position corresponding to position 206; R at a position corresponding to position 209; N at a position corresponding to position 212; S at a position corresponding to position 212; A at a position corresponding to position 213; M at a

position corresponding to position 213; N at a position corresponding to position 213; H at a position corresponding to position 215; M at a position corresponding to position 215; A at a position corresponding to position 219; I at a position corresponding to position 219; K at a position corresponding to position 219; S at a position corresponding to position 219; H at a position corresponding to position 220; I at a position corresponding to position 220; L at a position corresponding to position 220; V at a position corresponding to position 220; Q at a position corresponding to position 221; G at a position corresponding to position 222; F at a position corresponding to position 232; G at a position corresponding to position 233; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; A at a position corresponding to position 235; R at a position corresponding to position 236; C at a position corresponding to position 237; E at a position corresponding to position 237; H at a position corresponding to position 237; Q at a position corresponding to position 237; T at a position corresponding to position 237; E at a position corresponding to position 238; H at a position corresponding to amino acid position 238; S at a position corresponding to position 238; A at a position corresponding to position 240; Q at a position corresponding to position 240; I at a position corresponding to position 247; A at a position corresponding to position 248; V at a position corresponding to position 249; G at a position corresponding to position 257; T at a position corresponding to position 257; R at a position corresponding to position 257; N at a position corresponding to position 258; S at a position corresponding to position 258; P at a position corresponding to position 259; M at a position corresponding to position 260; Y at a position corresponding to position 260; A at a position corresponding to position 261; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position corresponding to position 263; T at a position corresponding to position 267; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; D at a position corresponding to position 272; T at a position corresponding to position 272; H at a position corresponding to position 273; Y at a position corresponding to position 273; F at a position corresponding to position 274; D at a position corresponding to position 276; H at a position corresponding to position 276; M at a position corresponding to position 276; R at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; A at a position corresponding to position 277; E at a position corresponding to position 277; H at a position corresponding to position 277; K at a position corresponding to position 277; M at a position corresponding to position 277; N at a position corresponding to position 277; Q at a position corresponding to position 277; R at a position corresponding to position 277; S at a position corresponding to position 277; T at a position corresponding to position 277; E at a position corresponding to position 278; F at a position corresponding to position 278; G at a position corresponding to position 278; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; H at a position corresponding to position 279; M at a position corresponding to position 282; S at a position corresponding to position 283; H at a position corresponding to position 285; T at a position corresponding to position 287; S at a position corresponding to position 289; S at a position corresponding to position 291; V at a position corresponding to position 291; C at a position corresponding to position 292; F at a position corresponding to position 292; H at a

position corresponding to position 292; K at a position corresponding to position 292; R at a position corresponding to position 292; V at a position corresponding to position 292; A at a position corresponding to position 293; C at a position corresponding to position 293; D at a position corresponding to position 293; F at a position corresponding to position 293; K at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; Y at a position corresponding to position 293; G at a position corresponding to position 298; E at a position corresponding to position 305; G at a position corresponding to position 307; D at a position corresponding to position 308; G at a position corresponding to position 308; K at a position corresponding to position 308; N at a position corresponding to position 308; R at a position corresponding to position 308; E at a position corresponding to position 309; G at a position corresponding to position 309; H at a position corresponding to position 309; L at a position corresponding to position 309; M at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; S at a position corresponding to position 309; T at a position corresponding to position 309; V at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; Q at a position corresponding to position 310; S at a position corresponding to position 310; A at a position corresponding to position 313; G at a position corresponding to position 313; H at a position corresponding to position 313; K at a position corresponding to position 313; P at a position corresponding to position 313; R at a position corresponding to position 313; T at a position corresponding to position 313; Y at a position corresponding to position 313; with S at a position corresponding to position 314; Y at a position corresponding to position 314; A at a position corresponding to position 315; H at a position corresponding to position 315; Y at a position corresponding to position 315; A at a position corresponding to position 317; I at a position corresponding to position 317; K at a position corresponding to position 317; N at a position corresponding to position 317; Q at a position corresponding to position 317; R at a position corresponding to position 317; S at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; H at a position corresponding to position 318; K at a position corresponding to position 318; M at a position corresponding to position 318; R at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 321; S at a position corresponding to position 321; N at a position corresponding to position 324; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 325; L at a position corresponding to position 326; V at a position corresponding to position 326; C at a position corresponding to position 328; G at a position corresponding to position 328; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; S at a position corresponding to position 335; A at a

position corresponding to position 347; G at a position corresponding to position 347; S at a position corresponding to position 347; M at a position corresponding to position 349; R at a position corresponding to position 349; S at a position corresponding to position 351; V at a position corresponding to position 353; with H at a position corresponding to position 356; S at a position corresponding to position 356; E at a position corresponding to position 359; H at a position corresponding to position 359; T at a position corresponding to position 359; A at a position corresponding to position 367; G at a position corresponding to position 367; K at a position corresponding to position 367; S at a position corresponding to position 367; A at a position corresponding to position 368; E at a position corresponding to position 368; K at a position corresponding to position 368; L at a position corresponding to amino acid position 368; M at a position corresponding to amino acid position 368; R at a position corresponding to position 368; T at a position corresponding to amino acid position 368; H at a position corresponding to position 369; R at a position corresponding to position 369; F at a position corresponding to position 371; H at a position corresponding to position 371; K at a position corresponding to position 371; L at a position corresponding to position 371; R at a position corresponding to position 371; S at a position corresponding to position 371; M at a position corresponding to position 373; H at a position corresponding to position 374; P at a position corresponding to position 374; A at a position corresponding to position 375; G at a position corresponding to position 375; K at a position corresponding to position 375; R at a position corresponding to position 375; D at a position corresponding to position 376; E at a position corresponding to position 376; Q at a position corresponding to position 376; R at a position corresponding to position 376; T at a position corresponding to position 376; V at a position corresponding to position 376; Y at a position corresponding to position 376; D at a position corresponding to position 377; E at a position corresponding to position 377; H at a position corresponding to position 377; K at a position corresponding to position 377; P at a position corresponding to position 377; R at a position corresponding to position 377; S at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; S at a position corresponding to position 381; I at a position corresponding to position 383; K at a position corresponding to position 383; L at a position corresponding to position 383; S at a position corresponding to position 383; A at a position corresponding to position 385; Q at a position corresponding to position 385; V at a position corresponding to position 385; A at a position corresponding to position 389; G at a position corresponding to position 389; L at a position corresponding to position 389; K at a position corresponding to position 389; Q at a position corresponding to position 389; S at a position corresponding to position 389; A at a position corresponding to position 392; F at a position corresponding to position 392; M at a position corresponding to position 392; Q at a position corresponding to position 392; R at a position corresponding to position 392; V at a position corresponding to position 392; F at a position corresponding to position 393; M at a position corresponding to position 393; A at a position corresponding to position 395; H at a position corresponding to position 395; R at a position corresponding to position 395; A at a position corresponding to position 396; H at a position corresponding to position 396; Q at a position corresponding to position 396; S at a position corresponding to position 396; K at a position corresponding to position 399; M at a position corresponding to position 399; T at a position corresponding to position 399; V at a position corresponding to position 399; W at a position corresponding to position 399; A at a position corresponding to position 401; E at a position corresponding to position 401; A at a position

corresponding to position 404; G at a position corresponding to position 405; F at a position corresponding to position 406; N at a position corresponding to position 406; A at a position corresponding to position 407; D at a position corresponding to position 407; E at a position corresponding to position 407; F at a position corresponding to position 407; H at a position corresponding to position 407; Q at a position corresponding to position 407; P at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; Q at a position corresponding to position 412; R at a position corresponding to position 412; V at a position corresponding to position 412; L at a position corresponding to position 416; E at a position corresponding to position 418; L at a position corresponding to position 418; P at a position corresponding to position 418; R at a position corresponding to position 418; V at a position corresponding to position 418; F at a position corresponding to position 419; H at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; Y at a position corresponding to position 419; A at a position corresponding to position 421; H at a position corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; G at a position corresponding to position 425; K at a position corresponding to position 425; Q at a position corresponding to position 427; T at a position corresponding to position 427; L at a position corresponding to position 428; A at a position corresponding to position 431; G at a position corresponding to position 431; E at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; L at a position corresponding to position 431; N at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; A at a position corresponding to position 433; H at a position corresponding to position 433; I at a position corresponding to position 433; K at a position corresponding to position 433; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; W at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; A at a position corresponding to position 438; D at a position corresponding to position 438; E at a position corresponding to position 438; L at a position corresponding to position 438; N at a position corresponding to position 438; T at a position corresponding to position 438; A at a position corresponding to position 439; C at a position corresponding to position 439; K at a position corresponding to position 439; P at a position corresponding to position 439; Q at a position corresponding to position 439; T at a position corresponding to position 439; V at a position corresponding to position 439; D at a position corresponding to position 440; H at a position corresponding to position 440; M at a position corresponding to position 440; P at a position corresponding to position 440; R at a position corresponding to position 440; S at a position corresponding to position 440; A at a position corresponding to position 441; F at a position corresponding to position 441; C at a position corresponding to position 442; G at a position corresponding to position 442; R at a position corresponding to position 442; A at a position corresponding to position 443; E at a position corresponding to position 443; F at a position corresponding to position 443; G at a position

corresponding to position 443; M at a position corresponding to position 443; N at a position corresponding to position 443; E at a position corresponding to position 444; H at a position corresponding to position 444; V at a position corresponding to position 444; H at a position corresponding to position 445; M at a position corresponding to position 445; N at a position corresponding to position 445; P at a position corresponding to position 445; Q at a position corresponding to position 445; S at a position corresponding to position 445; T at a position corresponding to position 445; V at a position corresponding to position 445; W at a position corresponding to position 445; A at a position corresponding to position 446; M at a position corresponding to position 446; W at a position corresponding to position 446; D at a position corresponding to position 447; E at a position corresponding to position 447; G at a position corresponding to position 447; I at a position corresponding to position 447; N at a position corresponding to position 447; P at a position corresponding to position 447; Q at a position corresponding to position 447; T at a position corresponding to position 447, and/or replacement with V at a position corresponding to position 447, each with reference to amino acid positions set forth in SEQ ID NO:3.

~~[0271]~~(167) Exemplary of such modified PH20 polypeptides are any having the sequence of amino acids set forth in any of SEQ ID NOS: 74-855, or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:74-855 and contains the amino acid replacement and exhibits hyaluronidase activity.

~~[0272]~~(168) Any of the above modified PH20 polypeptides provided herein can exhibit altered, such as improved or increased, properties or activities compared to the corresponding PH20 polypeptide not containing the amino acid modification (e.g., amino acid replacement). For example, the altered activities or properties can be an increased catalytic activity and/or an increased stability under denaturing conditions.

~~[0273]~~(169) a. Increased Activity

~~[0274]~~(170) Provided herein are modified or variant PH20 polypeptides that contain one or more amino acid replacements in a PH20 polypeptide and that exhibit increased hyaluronidase activity compared to the corresponding PH20 polypeptide not containing the amino acid replacement(s), for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. In particular, the modified or variant PH20 polypeptides provided herein exhibit increased hyaluronidase activity compared to the corresponding PH20 polypeptide not containing the amino acid replacement, for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 and in particular the PH20 polypeptide set forth in SEQ ID NO:3.

~~[0275]~~(171) The modified PH20 polypeptide can exhibit hyaluronidase activity that is at least or about at least or 120%, 130%, 135%, 140%, 145%, 150%, 160%, 170%, 180%, 200%, 250%, 300%, 350%, 400%, 500%, 1500%, 2000%, 3000%, 4000%, 5000% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid replacement(s), for example the PH20 polypeptide set forth in any of any of SEQ ID NOS: 2, 3, 6-66, 68-72,

856-861, 869 or 870 or a variant thereof, under the same conditions. For example, the hyaluronidase activity is increased at least or about at least 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 200-fold, 300-fold, 400-fold or more.

~~[0276]~~(172) In particular examples, the modified PH20 polypeptides contain an amino acid replacement at one or more amino acid positions identified as being associated with increased hyaluronidase activity. As described herein, such positions have been identified using mutagenesis and selection or screening methods to identify those positions that result in increased hyaluronidase activity. The PH20 polypeptide also can contain other modifications, such as other amino acid replacements, that alone are not associated with increased activity so long as the resulting modified PH20 polypeptide exhibits increased hyaluronidase activity compared to the PH20 not containing the amino acid modification(s), such as amino acid replacement(s). The modified PH20 polypeptide provided herein can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, or more amino acid replacements. Additional modifications, such as insertions or deletions, also can be included. The amino acid replacement can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacement(s) can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

~~[0277]~~(173) For example, the modified PH20 polypeptides provided herein contain an amino acid replacement (substitution) at one or more amino acid positions corresponding to positions 1, 12, 15, 24, 26, 27, 29, 30, 31, 32, 33, 37, 39, 46, 48, 52, 58, 63, 67, 68, 69, 70, 71, 72, 73, 74, 75, 84, 86, 87, 92, 93, 94, 97, 118, 120, 127, 131, 135, 141, 142, 147, 148, 150, 151, 152, 155, 156, 163, 164, 165, 166, 169, 170, 174, 198, 206, 209, 212, 213, 215, 219, 233, 234, 236, 238, 247, 257, 259, 260, 261, 263, 269, 271, 272, 276, 277, 278, 282, 291, 293, 305, 308, 309, 310, 313, 315, 317, 318, 320, 324, 325, 326, 328, 347, 353, 359, 371, 377, 380, 389, 392, 395, 399, 405, 407, 409, 410, 418, 419, 421, 425, 431, 433, 436, 437, 438, 439, 440, 441, 442, 443, 445, 446 or 447 with reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at positions corresponding to replacement of Leucine (L) at position 1 (L1), V12, L15, F24, L26, G27, F29, D30, E31, P32, L33, L37, S39, I46, A48, G52, V58, Y63, I67, D68, S69, I70, T71, G72, V73, T74, V75, S84, G86, D87, A92, K93, K94, T97, T118, A120, D127, N131, E135, N141, V142, T147, E148, T150, E151, K152, Q155, E156, D163, F164, L165, V166, I169, K170, L174, G198, V206, K209, D212, D213, S215, N219, Q233, Q234, P236, A238, V247, P257, A259, K260, S261, L263, T269, I271, V272, Q276, V277, L278, S282, G291, T293, G305, S308, I309, M310, M313, S315, L317, L318, D320, E324, T325, I326, N328, Q347, I353, S359, A371, G377, F380, E389, E392, S395, Y399, T405, S407, K409, E410, D418, A419, D421, A425, D431, F433, P436, P437, M438, E439, T440, E441, E442, P443, I445, F446 or Y447 with reference to amino acid positions set forth in SEQ ID NO:3. Exemplary of such modified PH20 polypeptides are polypeptides that exhibit at least

1.5-fold or more the activity of the corresponding PH20 polypeptide not containing the amino acid replacement.

~~[0278]~~(174) Exemplary of amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited, replacement: with histidine (H) at a position corresponding to position 1; Q at a position corresponding to position 1; E at a position corresponding to position 12; T at a position corresponding to position 12; V at a position corresponding to position 15; E at a position corresponding to position 24; H at a position corresponding to position 24; E at a position corresponding to position 26; K at a position corresponding to position 26; K at a position corresponding to position 27; R at a position corresponding to position 27; E at a position corresponding to position 29; I at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; S at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; F at a position corresponding to position 37; N at a position corresponding to position 39; T at a position corresponding to position 39; R at a position corresponding to position 46; F at a position corresponding to position 48; H at a position corresponding to position 48; N at a position corresponding to position 48; Q at a position corresponding to position 52; K at a position corresponding to position 58; Q at a position corresponding to position 58; W at a position corresponding to position 63; V at a position corresponding to position 67; H at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 69; C at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; R at a position corresponding to position 71; S at a position corresponding to position 71; M at a position corresponding to position 72; Q at a position corresponding to position 72; H at a position corresponding to position 73; L at a position corresponding to position 73; W at a position corresponding to position 73; A at a position corresponding to position 74; C at a position

corresponding to position 74; G at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; F at a position corresponding to position 75; L at a position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; R at a position corresponding to position 84; A at a position corresponding to position 86; C at a position corresponding to position 87; T at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; R at a position corresponding to position 93; T at a position corresponding to position 93; R at a position corresponding to position 94; G at a position corresponding to position 97; Q at a position corresponding to position 118; F at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; H at a position corresponding to position 127; N at a position corresponding to position 127; G at a position corresponding to position 131; R at a position corresponding to position 131; V at a position corresponding to position 131; D at a position corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135, with H at a position corresponding to position 141; Y at a position corresponding to position 141; R at a position corresponding to position 142; R at a position corresponding to position 147; V at a position corresponding to position 147; K at a position corresponding to position 148; G at a position corresponding to position 150; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; R at a position corresponding to position 151; R at a position corresponding to position 152; G at a position corresponding to position 155; K at a position corresponding to position 155; D at a position corresponding to position 156; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; R at a position corresponding to position 163; M at a position corresponding to position 164; D at a position corresponding to position 165; N at a position corresponding to position 165; A at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; Q at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; Y at a position corresponding to position 166; L at a position corresponding to position 169; R at a position corresponding to position 170; K at a position corresponding to position 174; D at a position corresponding to position 198; K at a position corresponding to position 206; L at a position corresponding to position 206; N at a position corresponding to position 212; M at a position corresponding to position 213; N at a position corresponding to position 213; M at a position corresponding to position 215; S at a position corresponding to position 219; K at a position corresponding to position 233; R at a position corresponding to position 233; M at a position corresponding to position 234; R at a position corresponding to position 236; E at a position corresponding to position 237; S at a position corresponding to position 238; I at a position corresponding to position 247; T at a position corresponding to position 257; P at a position corresponding to position 259; Y at a position corresponding to position 260; K at a position corresponding to position 261; N at a position corresponding to position 261; K at a position corresponding to position 263; R at a position

corresponding to position 263; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; T at a position corresponding to position 272; D at a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; K at a position corresponding to position 277; R at a position corresponding to position 277; T at a position corresponding to position 277; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; M at a position corresponding to position 282; V at a position corresponding to position 291; A at a position corresponding to position 293; C at a position corresponding to position 293; F at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; E at a position corresponding to position 305; G at a position corresponding to position 308; N at a position corresponding to position 308; E at a position corresponding to position 309; L at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; T at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; K at a position corresponding to position 313; R at a position corresponding to position 313; H at a position corresponding to position 315; I at a position corresponding to position 317; K at a position corresponding to position 317; R at a position corresponding to position 317; M at a position corresponding to position 318; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 326; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to position 328; G at a position corresponding to position 347; S at a position corresponding to position 347; V at a position corresponding to position 353; with T at a position corresponding to position 359; R at a position corresponding to position 371; P at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; K at a position corresponding to position 389; M at a position corresponding to position 392; R at a position corresponding to position 395; M at a position corresponding to position 399; T at a position corresponding to position 399; W at a position corresponding to position 399; G at a position corresponding to position 405; D at a position corresponding to position 407; Q at a position corresponding to position 407; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; P at a position corresponding to position 418; F at a position corresponding to position 419; I at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; H at a position

corresponding to position 421; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; K at a position corresponding to position 425; A at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to position 431; V at a position corresponding to position 431; L at a position corresponding to position 433; R at a position corresponding to position 433; T at a position corresponding to position 433; V at a position corresponding to position 433; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; T at a position corresponding to position 438; V at a position corresponding to position 439; H at a position corresponding to position 440; R at a position corresponding to position 440; F at a position corresponding to position 441; R at a position corresponding to position 442; A at a position corresponding to position 443; M at a position corresponding to position 443; M at a position corresponding to position 445; P at a position corresponding to position 445; A at a position corresponding to position 446; D at a position corresponding to position 447; N at a position corresponding to position 447; and/or with Q at a position corresponding to position 447, each with reference to amino acid positions set forth in SEQ ID NO:3. The modified PH20 polypeptides can contain any one or more of the recited amino acid substitutions, in any combination, with or without additional modifications, so long at the PH20 polypeptide exhibits hyaluronidase activity, such as increased hyaluronidase activity compared to the PH20 polypeptide not containing the modification(s), for example, at least 1.5-fold increased hyaluronidase activity.

~~[0279]~~(175) In some examples, the modified PH20 polypeptides provided herein contain one or more amino acid replacement(s) at a position(s) corresponding to position(s) 24, 29, 31, 48, 58, 69, 70, 75, 84, 97, 165, 166, 271, 278, 317, 320, 325, and/or 326 with reference to positions set forth in SEQ ID NO:3. For example, exemplary amino acid replacements include, but are not limited to, replacement with: E at a position corresponding to position 24; E at a position corresponding to position 29; V at a position corresponding to position 31; N at a position corresponding to position 48; K at a position corresponding to position 58; Q at a position corresponding to position 58; A at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; A at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; H at a position corresponding to position 70; N at a position corresponding to position 70; R at a position corresponding to position 70; T at a position corresponding to position 70; V at a position corresponding to position 70; L at a position corresponding to position 75; T at a position corresponding to position 75; G at a position corresponding to position 84; G at a position corresponding to position 97; D at a position corresponding to position 165; L at a position corresponding to position 166; R at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 271; H at a position corresponding to position 278; R at a position corresponding to position 278; K at a position corresponding to position 317; K at a position corresponding to position 320; E at a position corresponding to position 325, with G at a position corresponding to position 325; K at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position

corresponding to position 325; V at a position corresponding to position 326; each with reference to amino acid positions set forth in SEQ ID NO:3. The modified PH20 polypeptides can contain any one or more of the recited amino acid substitutions, in any combination, with or without additional modifications, so long at the PH20 polypeptide exhibits hyaluronidase activity, such as increased hyaluronidase activity compared PH20 polypeptide not containing the modification(s), for example, at least 2.0-fold increased hyaluronidase activity.

~~[0280]~~(176) Exemplary modified PH20 polypeptides that exhibit increased activity compared to the unmodified PH20 polypeptide (e.g., set forth in SEQ ID NO:3) are any having the sequence of amino acids set forth in any of SEQ ID NOS: 73, 78, 86, 89, 91, 95, 96, 99, 100, 105, 106, 108, 109, 111, 112, 113, 115, 117, 118, 119, 120, 123-126, 128-136, 139-141, 149, 154, 155, 159, 164, 165, 167, 173, 178, 181, 191-193, 195-197, 199-205, 207-221, 225, 226, 228, 229, 231, 233, 237-239, 242, 247-254, 256, 257, 267, 269, 270, 277, 283, 293, 295, 296, 298, 300, 303, 308, 316, 318, 321, 322, 324, 325, 330, 334, 335, 338-340, 344, 348, 355, 367, 369, 371, 377, 384-388, 394, 398, 399, 401, 406-408, 410, 412, 414, 416, 419, 421-426, 428, 430, 431, 435, 448, 455, 456, 459, 462, 463, 465, 469, 478-480, 482, 484, 490, 493, 497, 501, 503, 505, 506-508, 510-512, 514, 518, 522, 523, 527, 531, 533, 537-543, 545, 551, 558, 559, 561, 563-566, 569, 572, 574, 576, 579, 581-583, 585, 587, 588, 594, 596, 602, 605, 606, 609, 613, 618-620, 624-634, 637, 640-644, 647, 648, 652, 657, 675, 695, 698, 699, 700, 712, 717, 725, 731, 732, 734, 738, 742, 746, 748-750, 757, 760, 762-765, 768-773, 775, 779, 782, 783, 786-789, 794-797, 799-801, 807, 814, 816, 819, 822, 825, 826, 830, 836, 838, 844, 847, 851, 853 or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 73, 78, 86, 89, 91, 95, 96, 99, 100, 105, 106, 108, 109, 111, 112, 113, 115, 117, 118, 119, 120, 123-126, 128-136, 139-141, 149, 154, 155, 159, 164, 165, 167, 173, 178, 181, 191-193, 195-197, 199-205, 207-221, 225, 226, 228, 229, 231, 233, 237-239, 242, 247-254, 256, 257, 267, 269, 270, 277, 283, 293, 295, 296, 298, 300, 303, 308, 316, 318, 321, 322, 324, 325, 330, 334, 335, 338-340, 344, 348, 355, 367, 369, 371, 377, 384-388, 394, 398, 399, 401, 406-408, 410, 412, 414, 416, 419, 421-426, 428, 430, 431, 435, 448, 455, 456, 459, 462, 463, 465, 469, 478-480, 482, 484, 490, 493, 497, 501, 503, 505, 506-508, 510-512, 514, 518, 522, 523, 527, 531, 533, 537-543, 545, 551, 558, 559, 561, 563-566, 569, 572, 574, 576, 579, 581-583, 585, 587, 588, 594, 596, 602, 605, 606, 609, 613, 618-620, 624-634, 637, 640-644, 647, 648, 652, 657, 675, 695, 698, 699, 700, 712, 717, 725, 731, 732, 734, 738, 742, 746, 748-750, 757, 760, 762-765, 768-773, 775, 779, 782, 783, 786-789, 794-797, 799-801, 807, 814, 816, 819, 822, 825, 826, 830, 836, 838, 844, 847, 851, 853 and contains the amino acid replacement and exhibits increased hyaluronidase activity compared to the corresponding unmodified polypeptide.

~~[0281]~~(177) b. Increased Stability

~~[0282]~~(178) Provided herein are PH20 polypeptides that exhibit increased stability. In particular, the PH20 polypeptides exhibit increased stability in vivo and/or in vitro. For example, the PH20 polypeptides can exhibit increased stability under various storage conditions. The modified PH20 polypeptides provided herein that exhibit increased stability display, among other parameters, increased resistance to denaturation conditions, including but not limited to, denaturation conditions caused by temperature (e.g., elevated temperature such as heat), agitation, no or low salt, and/or presence of excipients. Exemplary excipients include, but are not limited to,

antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents or sweeteners. For example, various excipients, such as preservatives, can act as protein denaturing agents. Modified PH20 polypeptides provided herein that exhibit increased protein stability exhibit reduced aggregation, reduced precipitation and/or increased activity when exposed to a denaturation condition compared to the corresponding PH20 not containing the amino acid replacement. For example, modified PH20 polypeptides provided herein exhibit at least or at least about or 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 200%, 250%, 300%, 350%, 400%~~)~~, 450%, 500% or more increased activity when exposed to a denaturation condition compared to the corresponding PH20 polypeptide not containing the amino acid replacement when exposed to the same denaturation condition.

~~[0283]~~(179) The PH20 polypeptides provided herein that exhibit increased stability are modified or variant PH20 polypeptides that contain an amino acid replacement (substitution), deletion or insertion or other modification. Typically, the PH20 polypeptides provided herein that exhibit increased stability contain one or more amino acid replacements in a PH20 polypeptide compared to the corresponding PH20 polypeptide not containing the amino acid replacement(s), for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. In particular, the modified or variant PH20 polypeptides provided herein exhibit increased stability compared to the corresponding PH20 polypeptide not containing the amino acid replacement, for example, the PH20 polypeptide set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 and in particular the PH20 polypeptide set forth in SEQ ID NO:3.

~~[0284]~~(180) In particular examples, the modified PH20 polypeptides contain an amino acid replacement at one or more amino acid positions identified as being associated with increased stability. As described herein, such positions can be identified using mutagenesis and selection or screening methods to identify those positions that result in stability (e.g., increased activity) of the polypeptide compared to the corresponding PH20 not containing the modification upon exposure to one or more denaturation conditions. The PH20 polypeptide also can contain other modifications, such as other amino acid replacements, that alone are not associated with conferring stability, so long as the resulting modified PH20 polypeptide exhibits increased stability under one or more denaturation conditions compared to the PH20 not containing the amino acid modification(s), such as amino acid replacement(s), and exhibits hyaluronidase activity. The modified PH20 polypeptide provided herein can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, or more amino acid replacements. Additional modifications, such as insertions or deletions, also can be included. The amino acid replacement can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacements can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

~~[0285]~~(181) Exemplary of modified PH20 polypeptides provided herein are PH20 polypeptides that exhibit increased stability upon exposure to phenol compounds, high temperature (heat), and/or lack of NaCl.

~~[0286]~~(182) i. Phenophiles

~~[0287]~~(183) Provided herein are modified PH20 polypeptides that exhibit increased stability in the presence of phenolic compounds. Multidose formulations must contain antimicrobial preservatives to protect them from microbial contamination. For parenteral drug products, including insulin and other therapeutic agents, the most common preservatives are phenolic compounds, such as phenol, metacresol (m-cresol), benzyl alcohol, and parabens including methylparaben and propylparaben. The preservatives typically must be present at sufficient concentrations to satisfy regulatory rules. For example, regulatory requirements assert that the antimicrobial efficacy of the formulation must satisfy the preservative efficacy test (PET) requirements of the target markets. Currently different regulatory agencies have different pharmacopeial criteria for antimicrobial effectiveness for pharmaceutical products designed for multiple dosing. The PET requirements of the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP) differ considerably, imposing additional constraints in developing multidose formulations. Table 4 shows the criteria for injectable drugs to meet USP and EP criteria. Typically, formulations that meet EP (EPA or EPB) anti-microbial requirements contain more preservative than those formulated only to meet USP anti-microbial requirements.

(184) TABLE-US-00004 TABLE 4 USP and EP requirement for antimicrobial effectiveness testing Europe United States EPB EPA Requirement Time point USP (Minimum) (Preferred) Bacterial Log 6 h 2 Reduction* 24 h 1 3 7 d 1.0 3 No recovery 14 d 3.0 No increase No recovery 28 d No increase No increase No recovery Fungal Log 7 d No increase 2 Reduction* 14 d No increase 1 No increase 28 d No increase No increase No increase *Log.sub.10 unit reduction from initial measured inoculum; No increase: not more than 0.5 log.sub.10 unit increase than previously measured value.

~~[0288]~~(185) Anti-microbial preservatives can interact with proteins resulting in aggregations and negative effects on stability. Thus, although a necessary component, preservatives pose a significant problem in the development of stable, multidose formulations of proteins because they typically induce aggregation of the protein in aqueous solution. In particular, increasing or high amounts of preservatives can negatively impact the stability of a protein, including effects on physical stability (aggregation or precipitation) that can impact protein activity. For example, to meet the EP preservative efficacy requirements, relatively high amounts of phenolic compounds, such as phenol or m-cresol, can be required, which can influence stability of the protein formulation. For example, preservatives such as phenol, m-cresol, and benzyl alcohol have been shown to induce aggregation of human growth hormone (Maa and Hsu (1996) Int. J. Pharm. 140:155-168), recombinant interleukin-1 receptor (Remmele (1998) Pharm. Res. 15:200-208), human insulin-like growth factor I (Fransson (1997) Pharm. Res. 14:606-612), ~~rhIFN-gamma.~~rhIFN-γ (Lam (1997) Pharm. Res. 14:725-729) and cytochrome c (Singh et al. (2011) J. Pharm Sci., 100:1679-89). The destabilizing effect that preservatives have on proteins in solution has been a limiting factor in the development of multidose formulations, and to date, most protein therapeutics have been formulated for single use only.

[0289](186) PH20 hyaluronidase, such as rHuPH20, rapidly loses activity in the presence of preservatives, likely due to unfolding of the protein and subsequent aggregate formation. For example, as shown in the Examples herein, preservatives reduce PH20 enzymatic activity, particularly at elevated temperatures (see also U.S. Provisional Appl. No. 61/520,962; and U.S. application Ser. Nos. 13/507,263 and 13/507,262). For example, following incubation with 0.4% m-cresol for 4 hours, PH20 (e.g., rHuPH20) retains only about 10% of its activity (see e.g., Example 5). When incubated in the presence of 0.1% phenol and 0.15% or 0.315% m-cresol for 6 days at 37.degree.° C., PH20 (e.g., rHuPH20) retains about 0% to 15% activity, depending on the presence of other excipients or amounts of other excipients in the formulation (see e.g., Examples 9 and 10). For example, the presence of a higher concentration of salt generally increases the stability of PH20. In particular, the melting temperature of PH20, such as rHuPH20, is reduced significantly when phenolic preservatives, such as m-Cresol, are added to the formulation. For example, the unfolding temperature of rHuPH20 is reduced from 44.degree.° C. to 24.degree.° C. The lower PH20 unfolding temperatures leads to increased PH20 aggregation, especially at elevated temperatures, and reduced enzyme activity. The destabilizing effect is likely due to the hydrophobic nature of the phenolic preservatives. The hydrophobicity of the phenolic compounds can lead to interaction with rHuPH20 through nonspecific binding to the protein, ultimately perturbing the structural integrity of rHuPH20. This translates to a significant loss of rHuPH20 enzymatic activity in the presence of preservatives.

[0290](187) The modified PH20 polypeptides provided herein that exhibit increased stability in the presence of phenolic preservatives exhibit more than 15% enzymatic activity in the presence of at least one phenolic preservative for at least 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks or more compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative). In some examples, the modified PH20 polypeptides provided herein exhibit at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of a phenolic preservative compared to in the absence of preservative. For example, the phenolic preservative compound can be phenol, metacresol (m-cresol), benzyl alcohol, and/or parabens including methylparaben or propylparaben.

[0291](188) In particular examples, the increased stability in the presence of preservative is exhibited under temperature conditions of between or about between 0.degree.° C. to 40.degree.° C., such as between or about between 2.degree.° C. to 6.degree.° C., 24.degree.° C. to 32.degree.° C. or 35.degree.° C. to 40.degree.° C., and generally at or about at 4.degree.° C. or 5.degree.° C., 30.degree.° C. or 37.degree.° C. It is understood that since high temperature also can have a destabilizing effect on PH20 activity (see below), the percentage of enzymatic activity of a modified PH20 polypeptide provided herein in the presence of preservative is greater at lower temperatures than at higher temperatures.

[0292](189) Generally, the modified PH20 polypeptides provided herein exhibit increased stability, and the noted enzymatic activities, in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms in a sample

of the composition. For example, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms such that at least a 1.0 log 10 unit reduction in bacterial organisms occurs at 7 days following inoculation. In some examples, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms such that, when tested in an antimicrobial preservative effectiveness test (APET), following inoculation of the composition with a microbial inoculum there is at least a 1.0 log 10 unit reduction in bacterial organisms at 7 days following inoculation, at least a 3.0 log 10 unit reduction of bacterial organisms at 14 days following inoculation, at least no further increase in bacterial organisms after 28 days following inoculation, and at least no increase in fungal organisms after 7 days following inoculation. In other examples, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of preservative that kills or inhibits the propagation of microbial organisms such that, when tested in an antimicrobial preservative effectiveness test (APET), following inoculation of the composition with a microbial inoculum there is at least a 1.0 log 10 unit reduction of bacterial organisms at 24 hours following inoculation, at least a 3.0 log 10 unit reduction of bacterial organisms at 7 days following inoculation, no further increase in bacterial organisms after 28 days following inoculation, at least a 1.0 log 10 unit reduction of fungal organisms at 14 days following inoculation, and at least no further increase in fungal organisms after 28 days following inoculation. In yet another example, the modified PH20 polypeptides provided herein exhibit increased stability in the presence of an anti-microbial effective amount of the preservative that kills or inhibits the propagation of microbial organisms such that, when tested in an antimicrobial preservative effectiveness test (APET), following inoculation of the composition with a microbial inoculum there is at least a 2.0 log 10 unit reduction of bacterial organisms at 6 hours following inoculation, at least a 3.0 log 10 unit reduction of bacterial organisms at 24 hours following inoculation, no recovery of bacterial organisms after 28 days following inoculation of the composition with the microbial inoculum, at least a 2.0 log 10 unit reduction of fungal organisms at 7 days following inoculation, and at least no further increase in fungal organisms after 28 days following inoculation.

~~[0293]~~(190) For example, the modified PH20 polypeptides provided herein exhibit increased stability, and above recited enzymatic activity, in the presence of a total amount of one or more phenolic preservative agents as a percentage (%) of mass concentration (w/v) that is or is between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive.

~~[0294]~~(191) Generally, modified PH20 polypeptides provided herein exhibit increased stability in the presence of m-cresol and/or phenol. For example, modified PH20 polypeptides provided herein exhibit increased stability in the presence of m-cresol in an amount as a % of mass concentration (w/v) in a formulation containing the modified PH20 polypeptide of between or about between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4%. In other examples, modified PH20 polypeptides provided herein exhibit increased stability in the presence of phenol in an amount at a % of mass concentration (w/v) in a formulation containing the modified PH20 polypeptide of between or about between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% m-cresol. In further examples, modified PH20

polypeptides provided herein exhibit increased stability in the presence of phenol and m-cresol in an amount as a % of mass concentration (w/v) in a formulation containing the modified PH20 polypeptide of between or about between 0.05% to 0.25% phenol and between or about between 0.05% to 0.3% m-cresol, between or about between 0.10% to 0.2% phenol and between or about between 0.6% to 0.18% m-cresol, between or about between 0.1% to 0.15% phenol and 0.8% t 0.15% m-cresol, between or about between 0.10% to 0.15% phenol and between or about between 0.06% to 0.09% m-cresol, or between or about between 0.12% to 0.18% phenol and between or about between 0.14% to 0.22% m-cresol.

~~[0295]~~(192) In examples herein, modified PH20 polypeptides exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of at least about between or between 0.3% to 0.4%, inclusive, m-cresol and/or phenol for at least 4 hours at 37~~.degree.~~° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of the preservative for the same time period and under the same conditions (except for the presence of preservative). For example, modified PH20 polypeptides exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of about or 0.4% m-cresol for at least 4 hours at 37~~.degree.~~° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of the preservative for the same time period and under the same conditions (except for the presence of preservative). Modified PH20 polypeptides provided herein also exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of at least about between or between 0.2% to 0.4%, inclusive, m-cresol and/or phenol for at least 1 day, 2 days, 3 days, 4 days, 5 days or 6 days at 37~~.degree.~~° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative). For example, modified PH20 polypeptides provided herein exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of about or 0.10% phenol and about or 0.15% m-cresol for at least 1 day, 2 days, 3 days, 4 days, 5 days or 6 days at 37~~.degree.~~° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative). In other examples, modified PH20 polypeptides provided herein exhibit more than 15%, such as at least 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more enzymatic activity in the presence of about or 0.315% m-cresol for at least 1 day, 2 days, 3 days, 4 days, 5 days or 6 days, generally for at least 6 days, at 37~~.degree.~~° C. compared to the enzymatic activity of the modified PH20 polypeptide in the absence of preservative for the same time period and under the same conditions (except for the presence of preservative).

~~[0296]~~(193) For example, such modified PH20 polypeptides provided herein that exhibit increased stability to phenol compounds contain an amino acid replacement (substitution) at one or more amino acid positions corresponding to positions 10, 12, 20, 22, 26, 34, 36, 46, 50, 52, 58, 68, 70, 74, 82, 83, 84, 86, 97, 127, 131, 138, 142, 143, 144, 166, 169, 174, 193, 195, 196, 204, 205, 206, 213, 219, 234, 237, 238, 240, 249, 261, 267, 277, 279, 291, 309, 310, 314, 315, 317, 318, 347, 367, 375, 376, 399, 401, 407, 416, 419, 421, 431, 433, 439, 440, 443 or 445 with

reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at one or more positions corresponding to replacement of (P) at position 10 (P10), V12, A20, S22, L26, D34, S36, I46, G50, G52, V58, D68, I70, T74, K82, I83, S84, Q86, T97, D127, N131, Q138, V142, Q143, L144, V166, I169, L174, H193, K195, K196, F204, N205, V206, D213, N219, Q234, V237, A238, T240, E249, S261, A267, ~~V277, K279~~V277K279, G291, I309, M310, K314, S315, L317, Q347, P367, E375, K376, Y399, S401, S407, D416, A419, D421, D431, F433, E439, T440, P443 or I445 with reference to amino acid positions set forth in SEQ ID NO:3.

~~[0297]~~(194) Exemplary of amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited to, replacement with: glycine (G) at a position corresponding to position 10; K at a position corresponding to position 12; S at a position corresponding to position 20; T at a position corresponding to position 22; M at a position corresponding to position 26; W at a position corresponding to position 34; N at a position corresponding to position 36; L at a position corresponding to position 46; M at a position corresponding to position 50; T at a position corresponding to position 52; S at a position corresponding to position 52; C at a position corresponding to position 58; K at a position corresponding to position 58; R at a position corresponding to position 58; N at a position corresponding to position 58; Y at a position corresponding to position 58; P at a position corresponding to position 58; H at a position corresponding to position 58; P at a position corresponding to position 68; V at a position corresponding to position 70; E at a position corresponding to position 74; L at a position corresponding to position 82; N at a position corresponding to position 82; V at a position corresponding to position 83; Q at a position corresponding to position 83; S at a position corresponding to position 83; G at a position corresponding to position 83; N at a position corresponding to position 84; A at a position corresponding to position 86; K at a position corresponding to position 86; E at a position corresponding to position 97; L at a position corresponding to position 97; R at a position corresponding to position 127; R at a position corresponding to position 131; L at a position corresponding to position 138; K at a position corresponding to position 142; N at a position corresponding to position 142; P at a position corresponding to position 142; S at a position corresponding to position 142; T at a position corresponding to position 142; G at a position corresponding to position 143; K at a position corresponding to position 143; T at a position corresponding to position 144; Q at a position corresponding to position 166; T at a position corresponding to position 166; L at a position corresponding to position 169; G at a position corresponding to position 174; N at a position corresponding to position 174; Q at a position corresponding to position 193; T at a position corresponding to position 195; N at a position corresponding to position 195; E at a position corresponding to position 196; R at a position corresponding to position 196; P at a position corresponding to position 204; A at a position corresponding to position 205; E at a position corresponding to position 205; I at a position corresponding to position 206; A at a position corresponding to position 213; I at a position corresponding to position 219; M at a position corresponding to position 234; T at a position corresponding to position 237; H at a position corresponding to position 238; Q at a position corresponding to position 240; V at a position corresponding to position 249; A at a position corresponding to position 261; K at a position corresponding to position 261; T at a position corresponding to position 267; K at a position corresponding to position 277; H at a position corresponding to position 279; V at a position corresponding to position 279; V at a position corresponding to position 291; E at a position corresponding to position 309; Q at a position corresponding to

position 310; Y at a position corresponding to position 314; Y at a position corresponding to position 315; N at a position corresponding to position 317; W at a position corresponding to position 317; D at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 375; R at a position corresponding to position 376; V at a position corresponding to position 399; E at a position corresponding to position 401; A at a position corresponding to position 407; L at a position corresponding to position 416; K at a position corresponding to position 419; H at a position corresponding to position 421; E at a position corresponding to position 431; T at a position corresponding to position 433; V at a position corresponding to position 433; C at a position corresponding to position 439; P at a position corresponding to position 440; G at a position corresponding to position 443; N at a position corresponding to position 445, each with reference to amino acid residue positions set forth in SEQ ID NO:3.

~~[0298]~~(195) The amino acid ~~replacements~~replacement(s) can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%~~)~~, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacement(s) can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

~~[0299]~~(196) Exemplary modified PH20 polypeptides that exhibit increased stability to phenol compounds compared to the unmodified PH20 polypeptide (e.g., set forth in SEQ ID NO:3) are any having the sequence of amino acids set forth in any of SEQ ID NOS: 83, 88, 93, 94, 101, 144, 148, 158, 171, 176, 175, 177, 178, 180, 182, 183, 184, 185, 194, 221, 240, 259, 260, 261, 262, 263, 264, 268, 270, 272, 307, 309, 327, 334, 341, 351, 352, 353, 356, 357, 358, 359, 361, 424, 426, 430, 434, 436, 443, 444, 445, 446, 447, 449, 450, 451, 454, 461, 467, 480, 487, 489, 492, 495, 504, 505, 509, 527, 544, 576, 589, 600, 603, 607, 612, 614, 647, 658, 683, 687, 733, 736, 741, 754, 763, 768, 781, 796, 797, 809, 818, 829 or 837 or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 83, 88, 93, 94, 101, 144, 148, 158, 171, 176, 175, 177, 178, 180, 182, 183, 184, 185, 194, 221, 240, 259, 260, 261, 262, 263, 264, 268, 270, 272, 307, 309, 327, 334, 341, 351, 352, 353, 356, 357, 358, 359, 361, 424, 426, 430, 434, 436, 443, 444, 445, 446, 447, 449, 450, 451, 454, 461, 467, 480, 487, 489, 492, 495, 504, 505, 509, 527, 544, 576, 589, 600, 603, 607, 612, 614, 647, 658, 683, 687, 733, 736, 741, 754, 763, 768, 781, 796, 797, 809, 818, 829 or 837 and contains the amino acid replacement, exhibits hyaluronidase activity and exhibits increased stability in the presence phenol compounds compared to the corresponding unmodified polypeptide.

~~[0300]~~(197) In particular, provided herein is a modified PH20 polypeptide that contains an amino acid replacement with P at a position corresponding to amino acid residue 204 with reference to SEQ ID NO:3. Typically, the modified PH20 polypeptide is a human polypeptide. For example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement F204P in a sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 69, 72 or 32-66, or a sequence of amino acids that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:3, 7, 69, 72 or 32-66 so long as the modified polypeptide contains the amino acid replacement corresponding to F204P. In other cases, the modified PH20 polypeptide is a non-human polypeptide. For

example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement F204P in a sequence of amino acids set forth in SEQ ID NO:10, 12, 14, 857, 859, 861 or 870 or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 10, 12, 14, 857, 859, 861 or 870 so long as the modified polypeptide contains the amino acid replacement corresponding to F204P. In a further example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement F205P in a sequence of amino acids set forth in SEQ ID NO:24 or Y204P in SEQ ID NO:31, or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:24 or 31. Exemplary of such a modified PH20 polypeptide is a polypeptide having the sequence of amino acids set forth in SEQ ID NO:449, or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:449 and contains the amino acid replacement F204P, exhibits increased hyaluronidase activity and exhibits increased stability to phenol compounds compared to the corresponding unmodified polypeptide (e.g., SEQ ID NO:3). In any of the above examples, the modified PH20 polypeptide that contains an amino acid replacement with P at a position corresponding to amino acid residue 204 with reference to SEQ ID NO:3 does not have the sequence of amino acids set forth in SEQ ID NO:15-22, 28 or 29.

~~[0301]~~(198) In another example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement at a position corresponding to amino acid residue 58 with reference to SEQ ID NO:3. Exemplary of amino acid replacements are replacement with lysine (K) or with arginine (R) at a position corresponding to amino acid residue 58 with reference to SEQ ID NO:3. Typically, the modified PH20 polypeptide is a human polypeptide. For example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement V58K or V58R in a sequence of amino acids set forth in any of SEQ ID NOS: 3, 7, 69, 72 or 32-66, or a sequence of amino acids that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:3, 7, 69, 72 or 32-66. In other cases, the modified PH20 polypeptide is a non-human polypeptide. For example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement V58K or V58R in a sequence of amino acids set forth in SEQ ID NO:10, 12, 14, 20, 22, 24, 29, 857, 859, 861 or 870 or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 10, 12, 14, 20, 22, 24, 29, 857, 859, 861 or 870. ~~En~~In a further example, provided herein is a modified PH20 polypeptide that contains an amino acid replacement A58R in a sequence of amino acids set forth in SEQ ID NO:16 or 31, or a sequence that exhibits at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:16 or 31. Exemplary of such a modified PH20 polypeptide is a polypeptide having the sequence of amino acids set forth in SEQ ID NO:182, or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:182, which contains the amino acid replacement V58R and exhibits increased hyaluronidase activity and exhibits increased stability in the presence of phenol compounds compared to the corresponding unmodified polypeptide (e.g., SEQ ID NO:3).

~~[0302]~~(199) ii. Thermophiles

[0303](200) At elevated temperatures, PH20 hyaluronidases can lose activity. Provided herein are modified PH20 polypeptides that exhibit increased stability at elevated temperatures of between or about between 30.degree.° C. to 45.degree.° C., inclusive, such as between or about between 35.degree.° C. to 42.degree.° C., in particular at or about 37.degree.° C. For example, provided herein are modified PH20 polypeptides that are stable at elevated temperatures greater than 32.degree.° C. such as 35.degree.° C. to 45.degree.° C., 37.degree.° C. to 42.degree.° C. and in particular at or about 37.degree.° C. for at least 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, at least 5 days, at least 6 days or at least 7 days. Modified PH20 polypeptides that exhibit stability at elevated temperatures can be used in applications where temperatures are elevated, can fluctuate or can increase. This can occur, for example, in methods of administration utilizing pumps or other continuous infusion devices.

[0304](201) In particular, modified PH20 polypeptides provided herein that exhibit stability at elevated temperatures exhibit increased hyaluronidase activity at elevated temperature compared to the corresponding PH20 polypeptide not containing the modification, e.g., amino acid replacement. The PH20 polypeptides can exhibit increased hyaluronidase activity upon incubation at elevated temperatures greater than 32.degree.° C. such as 35.degree.° C. to 45.degree.° C. or 37.degree.° C. to 42.degree.° C., in particular at or about 37.degree.° C. for at least 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, at least 5 days, at least 6 days or at least 7 days compared to the corresponding PH20 polypeptide not containing the modification incubated under the same conditions. For example, the hyaluronidase activity can be increased at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more compared to the corresponding PH20 polypeptide not containing the modification incubated under the same conditions. For example, the hyaluronidase activity can be increased at least 1.1-fold, 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or more compared to the corresponding PH20 polypeptide not containing the modification incubated under the same conditions.

[0305](202) In other examples, modified PH20 polypeptides provided herein that exhibit stability at elevated temperatures retain hyaluronidase activity at elevated temperatures compared to the activity of the modified PH20 polypeptide incubated at non-elevated temperatures under the same conditions (except for the differences in temperature). For example, modified PH20 polypeptides exhibit greater than or about 50%, such as greater than or at least 55%, 60%, 65%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the activity at elevated temperatures greater than 32.degree.° C. such as 35.degree.° C. to 45.degree.° C. or 37.degree.° C. to 42.degree.° C., in particular at or about 37.degree.° C. compared to the activity of the PH20 at non-elevated temperatures of between or about between 2.degree.° C. to 8.degree.° C. In some examples, modified PH20 polypeptides provided herein that exhibit stability at elevated temperatures exhibit increased activity at elevated temperatures compared to the activity of the modified PH20 polypeptide incubated at non-elevated temperatures under the same conditions (except for the difference in temperature). For example, modified PH20 polypeptides exhibit greater than or about 10% increased activity, such as greater than or at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more of activity at elevated temperatures greater than 32.degree.° C. such as 35.degree.° C. to 45.degree.° C. or 37.degree.° C. to 42.degree.° C., in particular at or about 37.degree.° C. compared to the activity of the PH20 at non-elevated temperatures of between or about between 2.degree.° C. to

8.degree.° C. For example, modified PH20 polypeptides exhibit greater than or at least about 1.1-fold the hyaluronidase activity, such as greater than or at least 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or more of activity at elevated temperatures greater than 32.degree.° C. such as 35.degree.° C. to 45.degree.° C. or 37.degree.° C. to 42.degree.° C., in particular at or about 37.degree.° C. compared to the activity of the PH20 at non-elevated temperatures of between or about between 2.degree.° C. to 8.degree.° C.

[0306](203) For example, such modified PH20 polypeptides provided herein that exhibit increased stability at elevated temperatures contain an amino acid replacement (substitution) at one or more amino acid positions corresponding to positions 1, 11, 12, 14, 20, 26, 29, 34, 50, 58, 70, 82, 83, 84, 86, 87, 140, 142, 143, 147, 152, 166, 167, 172, 174, 178, 193, 195, 206, 212, 213, 219, 233, 237, 240, 267, 277, 291, 292, 309, 313, 314, 317, 318, 347, 367, 368, 371, 374, 389, 392, 395, 396, 406, 419, 421, 439 or 443 with reference to amino acid positions set forth in SEQ ID NO:3. For example, the amino acid positions can be replacements at one or more positions corresponding to replacement of (L) at position 1 (L1), N11, V12, F14, A20, L26, F29, D34, G50, V58, I70, K82, I83, S84, Q86, D87, Q140, V142, Q143, T147, K152, V166, E167, G172, L174, N178, H193, K195, V206, D212, D213, N219, Q233, V237, T240, A267, V277, G291, E292, I309, M313, K314, L317, L318, Q347, P367, D368, A371, L374, E389, E392, S395, E396, L406, A419, D421, E439 or P443, with reference to amino acid positions set forth in SEQ ID NO:3. The resulting modified PH20 polypeptide exhibits increased stability at elevated temperatures greater than 32.degree.° C. such as 35.degree.° C. to 45.degree.° C., 37.degree.° C. to 42.degree.° C. and in particular at or about 37.degree.° C. for at least 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, at least 5 days, at least 6 days, at least 7 days or more.

[0307](204) Exemplary amino acid replacements in the modified PH20 polypeptides provided herein include, but are not limited, replacement with: R at a position corresponding to position 1; S at a position corresponding to position 11; I at a position corresponding to position 12; V at a position corresponding to position 14; S at a position corresponding to position 20; M at a position corresponding to position 26; with R at a position corresponding to position 29; W at a position corresponding to position 34; M at a position corresponding to position 50; K at a position corresponding to position 58; Q at a position corresponding to position 58; Q at a position corresponding to position 58; V at a position corresponding to position 70; L at a position corresponding to position 82; Q at a position corresponding to position 83; R at a position corresponding to position 84; A at a position corresponding to position 86; S at a position corresponding to position 87; K at a position corresponding to position 140; S at a position corresponding to position 142; T at a position corresponding to position 142; K at a position corresponding to position 143; S at a position corresponding to position 147; T at a position corresponding to position 152; T at a position corresponding to position 166; D at a position corresponding to position 167; A at a position corresponding to position 172; G at a position corresponding to position 174; N at a position corresponding to position 174; R at a position corresponding to position 178; Q at a position corresponding to position 193; T at a position corresponding to position 195; I at a position corresponding to position 206; S at a position corresponding to position 212; A at a position corresponding to position 213; I at a position corresponding to position 219; G at a position corresponding to position 233; T at a position corresponding to position 237; A at a position corresponding to position 240; Q at a

position corresponding to position 240; T at a position corresponding to position 267; E at a position corresponding to position 277; S at a position corresponding to position 291; H at a position corresponding to position 292; V at a position corresponding to position 292; S at a position corresponding to position 309; H at a position corresponding to position 313; S at a position corresponding to position 314; I at a position corresponding to position 317; T at a position corresponding to position 317; W at a position corresponding to position 317; R at a position corresponding to position 318; G at a position corresponding to position 347; A at a position corresponding to position 367; R at a position corresponding to position 368; S at a position corresponding to position 371; P at a position corresponding to position 374; A at a position corresponding to position 389; V at a position corresponding to position 392; A at a position corresponding to position 395; H at a position corresponding to position 396; N at a position corresponding to position 406; H at a position corresponding to position 419; K at a position corresponding to position 419; R at a position corresponding to position 421; S at a position corresponding to position 421; A at a position corresponding to position 439; C at a position corresponding to position 439; or G at a position corresponding to position 443, each with reference to amino acid residue positions set forth in SEQ ID NO:3.

~~[0308]~~(205) The amino acid ~~replacements~~replacement(s) can be in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto. For example, the replacement(s) can be in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72 or a variant thereof.

~~[0309]~~(206) Exemplary modified PH20 polypeptides that exhibit increased stability to phenol compounds compared to the unmodified PH20 polypeptide (e.g., set forth in SEQ ID NO:3) are any having the sequence of amino acids set forth in any of SEQ ID NOS: 79, 85, 87, 90, 93, 101, 114, 144, 171, 178, 181, 221, 259, 262, 269, 270, 282, 343, 356, 357, 359, 368, 395, 426, 429, 432, 434, 436, 441, 443, 444, 454, 460, 461, 467, 477, 487, 491, 492, 509, 525, 550, 554, 557, 584, 593, 599, 605, 611, 612, 617, 647, 658, 667, 676, 679, 709, 720, 723, 727, 740, 761, 763, 772, 773, 808, 809, or 829 or having a sequence of amino acids that exhibits at least 68%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 79, 85, 87, 90, 93, 101, 114, 144, 171, 178, 181, 221, 259, 262, 269, 270, 282, 343, 356, 357, 359, 368, 395, 426, 429, 432, 434, 436, 441, 443, 444, 454, 460, 461, 467, 477, 487, 491, 492, 509, 525, 550, 554, 557, 584, 593, 599, 605, 611, 612, 617, 647, 658, 667, 676, 679, 709, 720, 723, 727, 740, 761, 763, 772, 773, 808, 809, or 829 and contains the amino acid replacement, exhibits hyaluronidase activity and exhibits increased stability to elevated temperatures compared to the corresponding unmodified polypeptide.

~~[0310]~~(207) iii. Absence of Salt

~~[0311]~~(208) PH20 denatures in the presence of low salt or no salt. Thus, PH20 requires a high salt concentration of between or about between 140 mM to 200 mM to maintain stability. Other therapeutic agents, for example insulin, exhibit decreased solubility and increased crystallization/aggregation in the presence of high salt. Thus, the high salt requirements of PH20 can affect the solubility and/or activity of co-formulated therapeutic agents, while the presence of

low salt can decrease the activity of PH20. This can create problems for generating PH20 co-formulations.

[0312](209) Provided herein are modified PH20 polypeptides that exhibit increased stability in the presence of low concentrations of salt (e.g. NaCl) less than 100 mM, for example, less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less. Generally, the modified PH20 polypeptides provided herein exhibit stability in the presence of low concentrations of salt, for example, low concentrations of NaCl of between or about between 10 mM NaCl and 100 mM NaCl, such as between or about between 15 mM to 80 mM NaCl. The modified PH20 polypeptides provided herein that exhibit stability at low concentrations of salt, such as low concentrations of NaCl (i.e., less than 100 mM or less), exhibit increased hyaluronidase activity compared to the corresponding PH20 not containing the modification(s) (e.g., amino acid replacements). For example, modified PH20 polypeptides exhibit greater than or about 10% increased activity, such as greater than or at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more of activity at low concentrations of salt, such as low concentrations of NaCl (i.e., less than 100 mM), compared to the activity of the corresponding PH20 not containing the amino acid modification(s) (e.g., amino acid replacement(s) under the same conditions). For example, modified PH20 polypeptides exhibit greater than or at least about 1.1-fold the hyaluronidase activity, such as greater than or at least 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or more of activity at low concentrations of NaCl less than 100 mM compared to the activity of the corresponding PH20 not containing the amino acid modification(s) (e.g., amino acid replacement(s) under the same conditions.

[0313](210) 2. Inactive Mutants

[0314](211) Provided herein are modified PH20 polypeptides that contain one or more amino acid replacements in a PH20 polypeptide and that are inactive, whereby the polypeptides do not exhibit hyaluronidase activity or exhibit low or diminished hyaluronidase activity. The modified PH20 polypeptides provided herein that are inactive generally exhibit less than 20%, such as less than 10%, of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the polypeptide set forth in SEQ ID NO: 3 or 7. For example, modified PH20 polypeptides provided herein that are inactive exhibit less than 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.05% or less of the hyaluronidase activity of a wildtype or reference PH20 polypeptide, such as the corresponding polypeptide not containing the amino acid modification (e.g., amino acid replacement), for example, a polypeptide set forth in SEQ ID NO:3 or 7.

[0315](212) For example, provided herein are PH20 polypeptides that are inactive and that are modified, for example by amino acid replacement or substitution, compared to a wildtype or reference PH20 polypeptide. For example, a modified PH20 polypeptide provided herein that is inactive contains one or more amino acid replacements at position(s) corresponding to position 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 27, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 94, 95, 96, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132,

133, 134, 135, 136, 137, 138, 139, 143, 144, 145, 149, 150, 152, 153, 154, 155, 156, 157, 158, 159, 161, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 197, 198, 199, 200, 201, 202, 203, 204, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 278, 279, 280, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 331, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 408, 410, 411, 412, 413, 414, 415, 416, 417, 419, 420, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 434, 437, 438, 439, 440, 441, 442, 443, 444, or 447 with reference to amino acid positions set forth in SEQ ID NO:3, so long as the resulting modified PH20 polypeptide is inactive and exhibits less than 20%, and generally less than 10%, of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid replacement. Typically, the amino acid residue that is modified (e.g., replaced) at the position corresponding to any of the above positions in a PH20 polypeptide is an identical residue, a conservative residue or a semi-conservative amino acid residue to the amino acid residue set forth in SEQ ID NO:3.

[0316](213) Exemplary amino acid replacements at any of the above corresponding positions are set forth in Table 5. Reference to corresponding position in Table 5 is with reference to positions set forth in SEQ ID NO:3. It is understood that the replacements can be made in the corresponding position in another PH20 polypeptide by alignment therewith with the sequence set forth in SEQ ID NO:3 (see e.g., FIGS. 1 and 2), whereby the corresponding position is the aligned position. The amino acid replacement(s) can be at the corresponding position in a PH20 polypeptide as set forth in any of SEQ ID NOS: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto, so long as the resulting modified PH20 polypeptide is inactive. For example, the replacement(s) can be in a corresponding position in a human PH20 polypeptide, for example, any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a variant thereof. In particular, any one or more of the replacements are in SEQ ID NO:3, so long as the resulting modified PH20 polypeptide is inactive and exhibits less than 20%, and generally less than 10%, of the hyaluronidase activity of the PH20 polypeptide set forth in SEQ ID NO:3

(214) TABLE-US-00005 TABLE 5 Inactive Mutants Corres- Corres- Corres- ponding ponding ponding Posi- Posi- Posi- tionCorre- Corre- Corre- sponding sponding sponding Position Replacement tionPosition Replacement tionPosition Replacement 2 H K W Y 3 A G K P T V 4 D E F G L V P W Y 5 D G I L M N P Q R 6 E F T V Y 7 C D F G H N P Q R T I K L Q TR V W Y R S T W Y 8 D E G H N R S W 9 C D E G N P 10 F I L M Y R S W P 11 A C F I L P T W Y 12 G H W 13 E G I L M P T W Y V 14 A E G H K N P Q W 15 E F G K N P Q R S 16 A C D E F N P Q W P Q R S Y G H K

Y M P R S T Y 17 D E G H I L N P Q 18 C D F G H I L M P 19 A C F G H L N P Q R I L M P R S T V W Y Q I L M P Q S T V W Y Q S T V Y R S V W Y 20 D E F H K L N P R 21 A C D E G H I L M 22 C E G K P L N P R T H I L M R V Y R S T V W 23 A F L M N P R S T 25 D E F G H 27 C P R S T V I K L N 27 C V P R S T V Y 33 C D H N V Y 34 I L N S T V 35 A D G P R Y V S 36 C F V W Y 37 C E G N S 38 E G K L N Q R T W 39 C D F W 40 A D E G K 41 Q N R S T 41 Q V 42 D E H I K L M P Q 43 A E F G I K L Q R 44 A C F G H L M P Q R K L Q R V I L N Q R S T V V R S T W Y 45 A D F G P W 46 P W 47 V W 48 P 49 C D G H P 50 V 51 C F I M P T W Y 52 C E F W Y 53 A C D E G T W Y H L N P Q R S T W Y 54 D E G P R Y 55 A D G H N P Q R T 56 A C E G H Y P Q R T V I K L P V R Y R S T V W 57 A D F G I M P Q R 58 A 59 A E I L M M P Q R V P R T V V W W Y 60 A D F G H I L N P 61 A E F G H N P Q R 62 A C D F I I L N P Q N P Q R T K L M P Q S T V Y T W Y Q R S T V Y 63 C G P 64 A C D E F G H I K 65 A C D G H G H I K L I K N R L S P Q R S T V W S T V W Y V W 66 A C D E G I K L N 67 D E G P R T W 68 A C G I L I K L N P T W P V Y P S T V 69 N T 70 Q 71 P 72 C F H I P V W 73 P 75 D G P V W 76 A C F G I K L P Q 77 D E L P Q R T V 78 A D I M P K L P Q R R T V T Y R S T V W 79 A D F G H K N P S 80 A D E F G I K L M 81 A C E G H L K N P S W Y I K L M N L N P S V Y R S T V Y V W Y 82 Y E K 84 Y 85 A C D E F G H N Q S T 86 C P 87 P 88 A C E F G I K L M P R S T V Y 89 A D E G Q S T W Y 90 C G 91 D E F G H S T W Y I L T 92 E F H K P Q R W Y 94 G P 95 A C E F G Q R W Y H K L M P Q S V W Y 96 S V H P R S T W 98 P 99 C E G I N S T W P V W 100 C E F G N P R S T 101 A C F H I 102 P P R S T W K L M N 102 P W Q Y Q R S T 103 A E F G H I L Q R T 104 F P W 105 C M N I L Q R T V W Y 106 A C D F H L M N P 107 A C H K P Q S V W 108 D E F K L L M N P S Q S V W M P Q T S V W Y V Y 109 C D E L M R T W 110 F K L M P W 111 H I Q R T W W 112 C E G H L N P S 113 R V 114 I L P T V N P S 115 A C D F G H I K L 116 A C D E G H I L N 117 D G I K N H I K L M H I L N P Q R V S W R S V M R S V Y P Q S V W W 118 C D E G P R W Y 119 A K I L N P R 121 A C E F G R W Y P R H K L M P W Y 122 A C E F I K Q R S 123 A C D E H L M P Q 124 C D E F N T V K Q R S T L M P Q R V S T V Y 125 C D G L N W 126 F H I L N P Y 127 K W P Y 128 E P 129 A C D E G H L P Q 130 C D G H L H L P Q S N S T S W Y T V W W Y 131 P 132 P 133 D E F G H L M N P R T V W 134 A C D F G H K P Q 135 P 136 P H K P Q R S W 137 F G H N P R W Y 138 V 139 P R W Y 143 C H P R S T 144 A E F I K 145 T W T P Q S V 145 T W Y 149 E 149 P 150 V 152 L 153 E F M P R T V 154 D E G P S T V W Y 155 P Y 156 P 157 A C D E G H I K L M P Q R S T V 158 D K P R Y 159 W Y 161 W 163 C P 164 A C D E G H N P Q 165 C H P T H N P Q R 166 D 167 V 168 A C D E F G K L P R S V W Y 169 A D F G H K

~~N P Q~~ 170 C D E G M ~~P W Y~~ 171 C D H M N K N P Q S P W Y R S W ~~S~~ Y T ~~Y~~ Y 172 D E I L P ~~Q T V W~~ 173 D E G H I 174 P Q T V W Y L M P S ~~174 P Y~~ V W Y 175 C D G K P ~~R S~~ 176 A C E F G ~~H I P Q~~ 177 A C D F G R S H I P Q S H L M Q R T V W S T V W ~~Q R S T V W~~ 178 E I L V W 180 A C E P R ~~S~~ 181 A C D E F S H I K L R S 182 A C D E H ~~N P Q R~~ 183 C D E G I ~~K N P Q~~ 184 A C D E F N P Q R S K N P Q R G H K L ~~S~~M T V Y ~~R~~ S V ~~M~~ P R S V 185 A D E F G ~~I K P R S~~ 186 A D G H I ~~K L N P~~ 187 A F G H I I K P R S K L N P Q L M N Q R T V W Y ~~Q~~ R S V W ~~R~~ S T V W Y 188 A C F G H ~~L M N P~~ 189 A E G H K ~~L M N P~~ 190 C E F G H L M N P Q L M N P R K L N Q ~~Q~~ R R S T V W ~~R~~ S T V W Y ~~R~~ S T V W 191 A E F G K ~~L M P Q~~ 192 C F G K L ~~M N P Q~~ 193 A D K L M L M P Q R M N P Q R P V ~~R~~ S T V W Y ~~R~~ V W Y 194 A C I L P ~~S T V~~ 195 S 197 C S T V 198 V W 199 E G H I K ~~L P R S~~ 200 A F G H K L P R S W L M P ~~W~~ Q R S W Y 201 A F L M N ~~P R S T~~ 202 A E F G H ~~K N P Q~~ 203 A D E G H P R S T V K N P Q R L M N ~~V W~~ Q R W V W Y ~~Q R~~ S T V 204 A C E G H ~~I K Q R~~ 206 C D F G P ~~Y~~ 207 A F G M P I K Q R S Y Q R S T ~~S~~ V T ~~V~~ W 208 D G P W 209 C P 210 A C D E G K M N P S T V W Y 211 C F G H I ~~K M P R~~ 212 A G H I K 213 P S K M P R S L M P V ~~213 P S S~~ W T V ~~W~~ W 214 A C D E G ~~H K N P~~ 215 C P 216 D E G H I H K N P R K L M N ~~R~~ P S T Y ~~P~~ Q R T V 217 A C G H P ~~Q S T V~~ 218 A I K L P ~~S V~~ 219 P Q S T V W S V 220 G K N P R ~~W~~ 221 D E H K P ~~R~~ 222 P Y W R 223 C D E G H ~~K L P Q~~ 224 A D E F G ~~M P Q R~~ 225 A D E G H K L P Q R M P Q R S K P Q R T S T V W Y ~~S~~ T W Y ~~R T~~ V W 226 A C D E F ~~G L N Q~~ 227 A F G H I ~~K L M P~~ 228 A E F G H G L N Q R K L M P Q L M N P R S T V W Y ~~Q~~ R T V W Y ~~R~~ S T W 229 E F G K L ~~P Q T V~~ 230 A E G H K ~~M N P R~~ 231 A C D F G P Q T V W M N P R S H I K L ~~W S~~ P T V ~~W Y P~~ W Y Q R S V 232 C G H K L ~~N P Q V~~ 233 D I P S T 234 A D E G H N P Q V Y N P S T ~~Y~~ V W 235 F L M R W ~~Y~~ 236 C I L N Q ~~T Y~~ 238 F G L P V Y T Y W Y 239 C F G H I ~~L P R S T~~ 240 E F G N W ~~Y~~ 241 A C D E G L P R S T Y I P R S ~~V W Y~~ T V W Y V W 242 A C D G I ~~L M P R~~ 243 C D F G H ~~L M P Q~~ 244 A D G I V ~~Y~~ L M P R S L M P Q R Y T V W ~~R~~ S W Y 245 A C F L P ~~Q R S T~~ 246 A C D E G ~~H I K L~~ 247 A C F H N Q R S T V H I K L M P Q R S ~~V M~~ T P S T V W ~~T~~ W Y 248 C D E G I ~~M P T~~ 249 A G H I K ~~M Q S Y~~ 250 C F G H K M P T M Q S Y L M N P Q R S T V W 251 D F G H K ~~P S T W~~ 252 A D E F G ~~H I K L~~ 253 A D E G H P S T W H I K L N L M N ~~N~~ Q R P S T Y ~~Q R~~ S W 254 C D E G I ~~K L P Q~~ 255 C D L P V ~~W~~ 256 C D E G [K L P Q R W T V W Y 257 D 258 L P V W 260 C P 261 P 262 A D E G H ~~I K Q R~~ 263 E F P Q W I K Q R S T V W Y 264 D E F G L ~~M R T V~~ 265 A D F G H ~~K L M~~ 266 A C G H M M R T V W K L M N Q P Q R ~~W~~ S T Y ~~N Q~~ R S ~~S T~~ V W 267 D G H I K ~~N R S W~~ 268 A C F G H ~~K L N P~~ 269 E K L M N N R S W K L N P Q P Q R ~~Q~~ S T V W 270 A C E F G ~~H I P Y~~ 271 A D E H K ~~T W~~

272 H L N P W H I P Y T W 273 A C D G I L P Q S 274 C E G H N Q W Y 275 A F G I K L P Q S V Q W Y L M Q T V W V W 276 F P W 278 M P 279 A C F G L W Y 280 D I M N R S T V W 281 A D G H I K N P Q 282 F L V W Y S T V W K N P Q R S V W 283 A C D F W 284 C D F W 284 C I P 285 K P R T V 286 A C D F H K M P T 287 A C D E G K M P T Y K L N P Y Q R S 288 D E F G H I K P R T 289 A C E G H L P Q R 290 D Q Y I K P R T L P Q R S Y 291 A C D E F M N T W 292 I L T 293 E N M N T W Y 294 A E G H K L N P Q 295 C G H I L N P T V 296 C F G I K L N P Q R N P T V Y M Q R S R T S T W Y T V W Y

297 C E H L N P Q R S 298 C E L M N P Q S T 299 A C D F G P Q R S T P Q S T W H L M T Y W Y P Q Y Y T 300 A C D E F L M N P 301 E G H K M N P Q R 302 C D E F G L M N P Q N P Q R S H L M P Q R S T V W S W Y R S T Y 303 A C D E F G K L M 304 A C D G I M N P Q 305 L P Q R S T V Y G K L M R W Y M N P Q S T V Y W Y T V Y 306 A C H I L V W Y 307 C I P 308 C F L M V V W Y W Y 310 C E F K L 311 C E F I L P V W 312 C E M V W P V W 313 C 314 C L W 315 C I V 316 E G I K L M P R S T 317 G P 318 C P W M P R S T V W Y 319 C E F G H I K M P 320 C P V 321 E M P I K M P Q R S V W Y 322 C D E G I L N P R S 323 A C E G H K N R S 324 C F P V W L N P R S K N R S T Y T V W T V 325 C R E G H N W 327 A E F G H N Q R S 329 C F G H I N W N Q R S T K L N Q T R V W Y R S T V W Y 330 A C D E G I L M N 331 A C D E F H K Q R 332 A C D E F I L M N P H K Q R S G H K L N R S V W T W Y P R S V W S T W Y N P R S T Y 333 G H I K P R S T W 334 A C D E G 335 F G H I K R S T W Y M N R S 335 L P V W Y 336 A E F G K 337 C F G I K 338 C D E F G N P R S T L M R T W H I K L P V W Y Y 336 A E F G K N P R S 337 C F G I K L M R T 338 C D E F G H I K L T V W Y W P R T V 339 D E F G H L N P S 340 A C D E F G H K P 341 A E G H K L N P S T G H K P R L M N T Q R V W Y R S T V W Q R S T V Y 342 D E F H K L M P Q 343 C D F I P W 344 F G H L M L M P Q R W N P Q R S T Y R S T W Y 345 A C E H K N Q R T 346 A D F G I K L M P 347 C F I P T N Q R T V K L M P R V W V Y R S T V W 348 C H I L P Q R T V 349 D F G P V W Y 350 A D E F H Q R T V W W Y K L M W Y N P Y R S T V Y 351 C D E F H N R W Y 352 A D E F G K M P Q 353 C F G H K N R W Y K M P Q R L M Q R S S T V W Y R S W 354 C D E G H I K L M 355 D F G H L M N P Q 356 C G K L P I K L M P M N P Q R R T V P W Q S V W Y R S T V W Y W 357 D E F G L M Q R 358 E H I K P Q R W 359 A F G L P M Q R Q R W W 360 A C E F G I K L M 361 A C E G M N P Q R 362 A C E G H I K L M P N P Q R S K L M N P Q R V S V W N P R S T V W 363 A C D E F G H I P 364 A C D E F G K L M 365 A C D E G G H I P Q G K L M P M N P Q R R S T V W P R S T V Y Q R S T W Y 366 A C E F G K M P Q 367 E F I L M Q V 368 C P W K M P Q R Q V T W 369 C E F I K L P Q V 370 A D E G H 371 P L P Q

V W K L N P 371 P W Q R S V Y 372 A D E F G H K L N 373 C P W 374 D E H K L N P R S T V W 375 C F P V Y 376 I P W 377 C I L V 378 D E F I L M Q T W 379 A C E F I L M W 380 C D E G Q M Q T W Y L M W R S Y 381 G L P W Y 382 E G H K L 383 G P M N P Q 383 G P R S T W Y 384 C F M Q S T 385 C L M P W Y 386 A C F G H T Y I L M N Q R S T V Y 387 C E F G H I L M N 388 C G P Q 389 F V I L M N V W Y 390 A C E F G H L N P 391 A D G H K 392 C P H L N P R N P Q R 392 C P R S S T V W Y S T V W Y 393 C P 394 A D E G I 395 C ; , [K N P Q 395 C;, / R S T V 396 C F G I P Y 397 A C E F G I L M P 398 A C E G H Y I L M P Q I L N P Q R T V R S T V W Y 399 D P 400 A D E F G I L M P 401 C F H K R I L M P Q W Y Q R S T V Y 402 A D E F L M P Q R 403 A C E G H K L M 404 C D F G H M P Q R S K L M N S P L M N R V T V W Y N P Q R T R V W Y 405 C I V 406 P R 408 A E F G I K L P R S T V W Y 410 W 411 D E F G 412 E H 413 H I K L P L P 414 A D E G H K R S T 415 C D E P K R S T 416 C S 417 A D E F G 419 D P H K M P 419 D P Q R 420 A D F G H K L N R 422 C D G H L M N Q 423 A D E F G K L N R S M N Q R S H L M P S Q T W Y Y R S Y Q R S T V W 424 A C E G H N Q R S 425 E L P W Y 426 C F M R N Q R S W Y 427 A C F L P V W Y 428 A C D E G H N R S 429 A D K L N V W Y H N R S Y P S T V Y W Y 430 A D E L M N S T V 431 P 432 C F I K L N S T V M P Y 434 H K P Q R W 437 T 438 Y W 439 N R 440 Q 441 R 442 M N S 443 D

[0317](215) 3. Additional Modifications and Conjugates

[0318](216) The modified PH20 polypeptides include those that contain chemical or posttranslational modifications. In some examples, modified PH20 polypeptides provided herein do not contain chemical or posttranslational modifications. Chemical and post-translational modifications include, but are not limited to, PEGylation, sialation, albumination, glycosylation, farnysylation, carboxylation, hydroxylation, phosphorylation, and other polypeptide modifications known in the art.

[0319](217) Also, in addition to any one or more amino acid modifications, such as amino acid replacements, provided herein, modified PH20 polypeptides provided herein can be conjugated or fused to any moiety using any method known in the art, including chemical and recombinant methods, provided the resulting polypeptide retains hyaluronidase activity. For example, in addition to any one or more amino acid modifications, such as amino acid replacements, provided herein, modified PH20 polypeptides provided herein also can contain other modifications that are or are not in the primary sequence of the polypeptide, including, but not limited to, modification with a carbohydrate moiety, a polyethylene glycol (PEG) moiety, a sialic acid moiety, an Fc domain from immunoglobulin G, or any other domain or moiety. For example, such additional modifications can be made to increase the stability or serum half-life of the protein.

[0320](218) In some instances, the domain or other moiety is a targeted agent, including any agent that targets the conjugate to one or more cell types by selectively binding to a cell surface

receptor or other cell surface moiety. For example, the domain or other moiety is a targeted agent that targets the conjugate to tumor cells. In such examples, a modified PH20 polypeptide, such as any provided herein, is linked directly or indirectly to a targeted agent. Such targeting agents include, but are not limited to, growth factors, cytokines, chemokines, antibodies, and hormones, or allelic variants, muteins, or fragments thereof so long as the targeting agent is internalized by a cell surface receptor. Exemplary, non-limiting, additional modifications are described below.

[0321](219) a. Decreased Immunogenicity

[0322](220) The modified PH20 polypeptides provided herein can be made to have decreased immunogenicity. Decreased immunogenicity can be effected by sequence changes that eliminate antigenic epitopes from the polypeptide or by altering post-translational modifications. One of skill in the art is familiar with methods of identifying antigenic epitopes in a polypeptide (see e.g., Liang et al. (2009) BMC Bioinformatics, 10:302; Yang et al. (2009) Rev. Med. Virol., 19:77-96). In some examples, one or more amino acids can be modified in order to remove or alter an antigenic epitope.

[0323](221) In another example, altering the glycosylation of a protein also can effect immunogenicity. For example, altering the glycosylation of the peptide is contemplated, so long as the polypeptides minimally contain at least N-acetylglucosamine at amino acid residues corresponding to amino acid residues set forth as N200, N333 and N358 of SEQ ID NO:3 or 7.

[0324](222) For example, the PH20 polypeptides can be modified such that they lack fucose, particularly bifucosylation. In particular, the PH20 polypeptides provided herein are not bifucosylated. This can be achieved by expressing and producing the PH20 polypeptide in host cells that do not effect bifucosylation. Fucose is a deoxyhexose that is present in a wide variety of organisms, including mammals, insects and plants. Fucosylated glycans are synthesized by fucosyl-transferases; see, e.g., Ma et al., Glycobiology, 16(12):158R-184R, (2006); Nakayama et al., J. Biol. Chem., 276:16100-16106 (2001); and Sturla et al., Glycobiology, 15(10):924-935 (2005). In humans, fucose frequently exists as a terminal modification to glycan structures, and the presence of fucose .alpha.1,6α1,6-linked to N-acetylglucosamine has been shown to be important in glycoprotein processing and recognition. In insects, N-glycan core structures exhibit bifucosylation with a 1,6α1,6- and .alpha.1,3α1,3-linkages. Insect cell core fucosylation with .alpha.1,3 α1,3-linkages generates a carbohydrate epitope that is immunogenic in humans (see, e.g., US Publication No. 20070067855). For example, PH20 polypeptides provided herein can be generated in host cells that are incapable of bifucosylating the polypeptide. Thus, while insect cells or other cells that bifucosylate can be used for expression of the polypeptides, typically mammalian cells, such as CHO cells, are used.

[0325](223) In some examples, defucosylated, or fucose-deficient PH20 polypeptides can be generated in insect cells with modified glycosylation pathways, through the use of baculovirus expression vectors containing eukaryotic oligosaccharide processing genes, thereby creating ""“mammalianized"” insect cell expression systems (see, e.g., U.S. Pat. No. 6,461,863). Alternatively, antigenicity can be eliminated by expression of PH20 polypeptides in insect cells lacking .alpha.1,3α1,3-fucosylatransferase (FT3) (see, e.g., US Publication No. 20070067855). In other examples, defucosylated or fucose-deficient PH20 polypeptides can be generated, for example, in cell lines that produce defucosylated proteins, including Lec 3Lec13 CHO cells

deficient in protein fucosylation (Ripka et al. Arch. Biochem. Biophys. 249:533-545 (1986); U.S. Pat. Pub. No. 2003/0157108; and WO 2004/056312), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, FUT8, knockout CHO cells (Yamane-Ohnuki et al. Biotech. Bioeng. 87: 614 (2004)).

~~[0326]~~(224) b. Conjugation to Polymers

~~[0327]~~(225) In some examples, the modified PH20 polypeptides provided herein are conjugated to polymers. Exemplary polymers that can be conjugated to the PH20 polypeptides, include natural and synthetic homopolymers, such as polyols (i.e., poly-OH), polyamines (i.e., poly-NH.sub.2) and polycarboxylic acids (i.e., poly-COOH), and further heteropolymers, i.e., polymers containing one or more different coupling groups, e.g., hydroxyl groups and amine groups. Examples of suitable polymeric molecules include polymeric molecules selected from among polyalkylene oxides (PAO), such as polyalkylene glycols (PAG), including polyethylene glycols (PEG), methoxypolyethylene glycols (mPEG) and polypropylene glycols, PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole (CDI-PEG), branched polyethylene glycols (PEGs), polyvinyl alcohol (PVA), polycarboxylates, polyvinylpyrrolidone, poly-D,L-amino acids, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextrans including carboxymethyl-dextrans, heparin, homologous albumin, celluloses, including methylcellulose, carboxymethylcellulose, ethylcellulose, hydroxyethylcellulose, carboxyethylcellulose and hydroxypropylcellulose, hydrolysates of chitosan, starches such as hydroxyethyl-starches and hydroxypropyl-starches, glycogen, agaroses and derivatives thereof, guar gum, pullulan, inulin, xanthan gum, carrageenan, pectin, alginic acid hydrolysates and bio-polymers.

~~[0328]~~(226) Typically, the polymers are polyalkylene oxides (PAO), such as polyethylene oxides, such as PEG, typically mPEG, which have few reactive groups capable of cross-linking~~.~~ Typically, the polymers are non-toxic polymeric molecules such as (methoxy)polyethylene glycol (mPEG) which can be covalently conjugated to the PH20 polypeptides (e.g., to attachment groups on the protein surface) using a relatively simple chemistry.

~~[0329]~~(227) Suitable polymeric molecules for attachment to the PH20 polypeptides include, but are not limited to, polyethylene glycol (PEG) and PEG derivatives such as ~~methoxy-polyethylene~~methoxypolyethylene glycols (mPEG), PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole

~~[0330]~~ (CDI-PEG), branched PEGs, and polyethylene oxide (PEO) (see e.g., Roberts et al., Advanced Drug Delivery Review 2002, 54:459-476; Harris and Zalipsky (eds.) ~~"~~"Poly(ethylene glycol), Chemistry and Biological Applications~~"~~" ACS Symposium Series 680, 1997; Mehvar et al., J. Pharm. Pharmaceut. Sci., 3(1):125-136, 2000; Harris and Chess (2003) Nat Rev Drug Discov. 2(3):214-21; and Tsubery, J Biol. Chem 279(37):38118-24, 2004). The polymeric molecule can be of a molecular weight typically ranging from about 3 kDa to about 60 kDa. In some embodiments the polymeric molecule that is conjugated to a PH20 polypeptide provided herein has a molecular weight of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 or more than 60 kDa.

[0331](228) Various methods of modifying polypeptides by covalently attaching (conjugating) a PEG or PEG derivative (i.e., ""PEGylation"") are known in the art (see e.g., U.S. 2006/0104968; U.S. Pat. NoNos. 5,672,662; U.S. Pat. No. 6,737,505; and U.S. 2004/0235734). Techniques for PEGylation include, but are not limited to, specialized linkers and coupling chemistries (see e.g., Roberts, Adv. Drug Deliv. Rev. 54:459-476, 2002), attachment of multiple PEG moieties to a single conjugation site (such as via use of branched PEGs; see e.g., Guiotto et al., Bioorg. Med. Chem. Lett. 12:177-180, 2002), site-specific PEGylation and/or mono-PEGylation (see e.g., Chapman et al., Nature Biotech. 17:780-783, 1999), and site-directed enzymatic PEGylation (see e.g., Sato, Adv. Drug Deliv. Rev., 54:487-504, 2002) (see, also, for example, Lu and Felix (1994) Int. J. Peptide Protein Res. 43:127-138; Lu and Felix (1993) Peptide Res. 6:140-6, 1993; Felix et al. (1995) Int. J. Peptide Res. 46:253-64; Benhar et al. (1994) J. Biol. Chem. 269:13398-404; Brumeanu et al. (1995) J Immunol. 154:3088-95; see also, Caliceti et al. (2003) Adv. DrugDelivDrug Deliv. Rev. 55(10):1261-77 and Molineux (2003) Pharmacotherapy 23 (8 Pt 2):3S-8S). Methods and techniques described in the art can produce proteins having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 PEG or PEG derivatives attached to a single protein molecule (see e.g., U.S. 2006/0104968).

[0332](229) Numerous reagents for PEGylation have been described in the art. Such reagents include, but are not limited to, N-hydroxysuccinimidyl (NHS) activated PEG, succinimidyl mPEG, mPEG2-N-hydroxysuccinimide, mPEG succinimidyl alpha-methylbutanoate, mPEG succinimidyl propionate, mPEG succinimidyl butanoate, mPEG carboxymethyl 3-hydroxybutanoic acid succinimidyl ester, homobifunctional PEG-succinimidyl propionate, homobifunctional PEG propionaldehyde, homobifunctional PEG butyraldehyde, PEG maleimide, PEG hydrazide, p-nitrophenyl-carbonate PEG, mPEG-benzotriazole carbonate, propionaldehyde PEG, mPEG butryaldehyde,butyraldehyde, branched mPEG2 butyraldehyde, mPEG acetyl, mPEG piperidone, mPEG methylketone, mPEG ""linkerless"" maleimide, mPEG vinyl sulfone, mPEG thiol, mPEG orthopyridylthioester, mPEG orthopyridyl disulfide, Fmoc-PEG-NHS, Boc-PEG-NHS, vinylsulfone PEG-NHS, acrylate PEG-NHS, fluorescein PEG-NHS, and biotin PEG-NHS (see e.g., Monfardini et al., Bioconjugate Chem. 6:62-69, 1995; Veronese et al., J. Bioactive Compatible Polymers 12:197-207, 1997; U.S. Pat. NoNos. 5,672,662; U.S. Pat. No. 5,932,462; U.S. Pat. No. 6,495,659; U.S. Pat. No. 6,737,505; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,179,337; U.S. Pat. No. 5,122,614; U.S. Pat. No. 5,324,844; U.S. Pat. No. 5,446,090; U.S. Pat. No. 5,612,460; U.S. Pat. No. 5,643,575; U.S. Pat. No. 5,766,581; U.S. Pat. No. 5,795,569; U.S. Pat. No. 5,808,096; U.S. Pat. No. 5,900,461; U.S. Pat. No. 5,919,455; U.S. Pat. No. 5,985,263; U.S. Pat. No. 5,990,237; U.S. Pat. No. 6,113,906; U.S. Pat. No. 6,214,966; U.S. Pat. No. 6,258,351; U.S. Pat. No. 6,340,742; U.S. Pat. No. 6,413,507; U.S. Pat. No. 6,420,339; U.S. Pat. No. 6,437,025; U.S. Pat. No. 6,448,369; U.S. Pat. No. 6,461,802; U.S. Pat. No. 6,828,401; U.S. Pat. No. 6,858,736; U.S. 2001/0021763; U.S. 2001/0044526; U.S. 2001/0046481; U.S. 2002/0052430; U.S. 2002/0072573; U.S. 2002/0156047; U.S. 2003/0114647; U.S. 2003/0143596; U.S. 2003/0158333; U.S. 2003/0220447; U.S. 2004/0013637; US 2004/0235734; U.S. 2005/0114037; U.S. 2005/0171328; U.S. 2005/0209416; EP 1064951; EP 0822199; WO 01076640; WO 0002017; WO 0249673; WO 9428024; WO 0187925; and WO 2005000360).

D. Methods for Identifying Modified Hyaluronan-Degrading Enzymes with Altered Properties or ActivitiesMETHODS FOR IDENTIFYING MODIFIED HYALURONAN-DEGRADING ENZYMES WITH ALTERED PROPERTIES OR ACTIVITIES

~~[0333]~~(230) Provided herein are methods for identifying a modified or variant hyaluronan-degrading enzyme, such as a modified hyaluronidase or modified PH20 polypeptide, that exhibits an altered activity or property compared to an unmodified hyaluronan-degrading enzyme. In particular, the methods provided herein can be used to screen for one or more modified hyaluronan-degrading enzymes, such as one or more modified hyaluronidase or PH20 polypeptide, that exhibits increased activity and/or increased stability in the presence of a denaturation agent or condition. For example, the methods can be used to identify a modified or variant hyaluronan-degrading enzyme, such as a modified or variant hyaluronidase or modified or variant PH20 polypeptide, that exhibits increased stability by virtue of increased resistance to denaturation conditions, including but not limited to, denaturation conditions caused by temperature (e.g., elevated temperature such as heat), agitation, no or low salt, presence of an excipient and/or a denaturing agent. Exemplary denaturing agents or excipients include, but are not limited to, antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents or sweeteners. For example, various excipients, such as preservatives, can act as protein denaturing agents. In the method, the activity also can be compared to an unmodified hyaluronan-degrading enzyme under the same denaturation condition, and a modified hyaluronan-degrading enzyme identified or selected that exhibits greater activity than the corresponding unmodified hyaluronan-degrading enzyme.

~~[0334]~~(231) In the method, one or more modified hyaluronan-degrading enzymes are provided. In some examples, a library of modified molecules is prepared. Methods of mutagenesis and generation of libraries or collections of variant molecules is described herein and is known to one of skill in the art using standard recombinant DNA techniques. In one example, the enzymes that are tested can be pooled and screened, whereby the method permits selection of only those enzymes that exhibit a desired activity. In another example, the tested enzymes can be physically separated and screened individually, such as by formatting in arrays, such as addressable arrays.

~~[0335]~~(232) In one aspect of the method, the modified hyaluronan-degrading enzymes are tested or screened for hyaluronidase activity in the presence and absence of one or more denaturation conditions or denaturing agent. After testing under both sets of conditions, the activities are assessed in order to identify modified hyaluronan-degrading enzymes that exhibit activity in the presence of the denaturation condition. The desired level or amount of activity selected as a cut-off in the methods can be empirically determined by the user, and is dependent on factors such as the particular hyaluronan-degrading enzyme, the desired application or use of the ~~hyaluronan-degrading~~hyaluronan-de grading enzyme, the particular denaturation condition or denaturing agent and other similar factors. Typically, a modified hyaluronan-degrading enzyme is identified that exhibits at least 5% or 10% of the activity in the presence of a denaturing agent or condition compared to in its absence, and generally at least 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more, for example at least 40% of the activity.

~~[0336]~~(233) Additionally or alternatively, the activity of the modified ~~hyaluronan-degrading~~hyaluronan-de grading enzyme in the presence of one or more denaturation conditions or denaturing agents is compared to the activity of the corresponding unmodified ~~hyaluronan-degrading~~hyaluronan-de grading enzyme in the presence of the same denaturation agent(s) or condition(s). In such examples, it is understood that the activity of the modified and unmodified enzyme are tested under the same conditions (e.g., time, temperature, composition), except for the difference in the particular enzyme tested (unmodified versus modified). A modified

hyaluronan-degrading enzyme is identified that exhibits greater activity, such as at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500% or more of the activity of the unmodified hyaluronan-degrading enzyme.

[0337](234) The method can be performed a plurality of times, whereby the steps of the method are repeated 1, 2, 3, 4, or 5 times. The method provided herein also is iterative. In one example, after the method is performed, any identified modified hyaluronan-degrading enzyme can be modified or further modified to increase or optimize the activity.

[0338](235) A description of the steps of the method and components of the method are provided in the subsections that follow.

[0339](236) 1. Hyaluronan-Degrading Enzymes and Libraries of Modified Hyaluronan-Degrading Enzymes

[0340](237) In the methods herein, one or more modified hyaluronan-degrading enzymes, such as a hyaluronidase or a PH20 polypeptide, are tested for a desired activity or property, such as increased stability (e.g., increased resistance to a denaturation condition). The modified hyaluronan-degrading enzyme can be modified compared to an unmodified hyaluronan-degrading enzyme, such as any hyaluronan-degrading enzyme known in the art. Hyaluronan-degrading enzymes are a family of enzymes that degrade hyaluronic acid, which is an essential component of the extracellular matrix and a major constituent of the interstitial barrier. Hyaluronan-degrading enzymes act to degrade hyaluronan by cleaving hyaluronan polymers, which are composed of repeating disaccharides units: D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc), linked together via alternating .beta.1β-1.fwdarw.4 and .beta.β-1.fwdarw.3 glycosidic bonds. By catalyzing the hydrolysis of hyaluronic acid, a major constituent of the interstitial barrier, hyaluronan-degrading enzymes lower the viscosity of hyaluronic acid, thereby increasing tissue permeability. Accordingly, hyaluronan-degrading enzymes for the uses and methods provided herein include any enzyme having the ability to catalyze the cleavage of a hyaluronan disaccharide chain or polymer. In some examples, the hyaluronan-degrading enzyme cleaves the .beta.β-1.fwdarw.4 glycosidic bond in the hyaluronan chain or polymer. In other examples, the hyaluronan-degrading enzyme catalyzes the cleavage of the .beta.β-1.fwdarw.3 glycosidic bond in the hyaluronan chain or polymer.

[0341](238) Hyaluronan-degrading enzymes include enzymes that are membrane-bound or that are soluble forms that are secreted from cells. Thus, where hyaluronan-degrading enzymes include a glycosylphosphatidylinositol (GPI) anchor signal sequence and/or are otherwise membrane-anchored or insoluble, such hyaluronan-degrading enzymes can be provided in soluble form by C-terminal truncation or deletion of all or a portion of the GPI anchor signal sequence to render the enzyme secreted and soluble. Thus, hyaluronan-degrading enzymes include C-terminally truncated variants, e.g., truncated to remove all or a portion of a GPI anchor signal sequence. Examples of such soluble hyaluronidases are soluble PH20 hyaluronides, such as any set forth in U.S. Pat. No. 7,767,429; U.S. Publication Nos. US 2004/0268425 and US 2010/0143457.

[0342](239) Exemplary hyaluronan-degrading enzymes are non-human animal or human hyaluronidases, bacterial hyaluronidases, hyaluronidases from leeches or chondroitinases that

exhibit hyaluronan-degrading activity, including soluble or truncated forms thereof that are active. Exemplary non-human animal hyaluronidases are any set forth in any of SEQ ID NOS: 8-31, 856-861, 869, 870, 871-886, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof. Exemplary human hyaluronidases are any set forth in any of SEQ ID NOS: 2, 3, 6, 7, 32-66, 68-72 or 887-890, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof, and in particular any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. Exemplary bacterial hyaluronidases are any set forth in any of SEQ ID NOS: 891-919 or mature, C-terminally truncated variants that are soluble and active, or active forms thereof. Exemplary hyaluronidases from leeches are set forth in SEQ ID NO:920 or 921, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof. Exemplary chondroitinases that have hyaluronan-degrading enzyme activity are set forth in SEQ ID NO:922-924, or mature, C-terminally truncated variants that are soluble and active, or active forms thereof.

~~[0343]~~(240) For example, one or more modified PH20 polypeptides are tested for a desired activity or property, such as increased stability (e.g., increased resistance to a denaturation condition). The modified PH20 polypeptide can be modified compared to an unmodified PH20 polypeptide, such as any known PH20 polypeptide native, wildtype or reference polypeptide. For example, the modified PH20 polypeptide is modified compared to a full-length, soluble or active form of a PH20 polypeptide, such as any set forth in any of SEQ ID NOS: 3, 7, 32-66, 69 or 72, or a polypeptide that exhibits at least 85%, such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 3, 7, 32-66, 69 or 72. In particular examples of the method herein, the starting or unmodified PH20 polypeptide has the sequence of amino acids set forth in SEQ ID NO:3.

~~[0344]~~(241) Libraries or collections of modified hyaluronan-degrading enzymes can be screened. Hyaluronan-degrading enzymes can be modified by any process known to one of skill in the art that can alter the structure of a protein. Examples of modifications include replacement, addition, and deletion of one or more amino acids of the protein to form libraries or collections of modified hyaluronan-degrading enzymes. It is within the level of one of skill in the art to generate modified or variant proteins for use in the methods herein. Methods of mutagenesis are well known in the art and include, for example, site-directed mutagenesis such as for example QuikChange (Stratagene) or saturation mutagenesis. Mutagenesis methods include, but are not limited to, site-mediated mutagenesis, PCR mutagenesis, cassette mutagenesis, site-directed mutagenesis, random point mutagenesis, mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA, point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and many others known to persons of skill. In the methods herein, mutagenesis can be effected across the full length of a protein or within a region of a protein. The mutations can be made rationally or randomly.

~~[0345]~~(242) In some examples, the methods provided herein are performed such that the identity of each mutant protein is known a priori before the protein is tested. For example, the methods provided herein can be conducive to mutagenesis and screening or testing methods that are addressable. This can permit the ease of comparisons between the activities of tested proteins without the need for sequencing of identified proteins. For example, site-directed mutagenesis

methods can be used to individually generate mutant proteins. Mutagenesis can be performed by the replacement of single amino acid residues at specific target positions one-by-one, such that each individual mutant generated is the single product of each single mutagenesis reaction. Mutant DNA molecules can be designed, generated by mutagenesis and cloned individually, such as in addressable arrays, such that they are physically separated from each other and each one is the single product of an independent mutagenesis reaction. The amino acids selected to replace the target positions on the particular protein being optimized can be either all of the remaining 19 amino acids, or a more restricted group containing only selected amino acids. In some methods provided herein, each amino acid that is replaced is independently replaced by 19 of the remaining amino acids or by less than 19 of the remaining amino acids, such as 10, 11, 12, 13, 14, 15, 16, 17 or 18 of the remaining amino acids.

~~[0346]~~(243) 2. Screening or Testing for a Desired Activity or Property

~~[0347]~~(244) The hyaluronidase activity or other activity of a composition containing a modified hyaluronan-degrading enzyme is screened or tested under conditions that expose the hyaluronan-degrading enzyme to a denaturation condition or a denaturing agent (presence of denaturation condition or denaturing agent). The denaturing condition or denaturing agent need not be a condition or agent that is completely deadly to the enzyme, but generally is any condition or agent that destabilizes enzyme activity over time. For example, the denaturation condition can be a condition caused by temperature (e.g., elevated temperature such as greater than or about or 30~~.degree.~~° C., for example, 30~~.degree.~~° C. to 42~~.degree.~~° C. such as or about 37~~.degree.~~° C.), agitation, no or low salt (e.g., NaCl), and/or caused by the presence of a denaturing agent, such as the presence of excipients (e.g., presence of preservatives).

~~[0348]~~(245) For purposes of selecting or identifying a modified hyaluronan-degrading enzyme that exhibits stability or increased stability under the denaturation condition, activity can be compared to activity of the modified hyaluronan-degrading enzyme in the absence of the denaturation condition and/or activity of the corresponding unmodified hyaluronan-degrading enzyme in the presence of the denaturation condition. For example, the modified hyaluronan-degrading enzyme also can be screened or tested under the same conditions, except not including a denaturing condition or denaturing agent (absence of denaturation condition or denaturing agent). If desired, the activity of the corresponding unmodified hyaluronan-degrading enzyme (e.g., the ~~hyaluronan-degrading~~hyaluronan-de grading enzyme not containing the amino acid ~~replacements~~replacement(s)) can also be tested under the same conditions that expose the hyaluronan-degrading enzyme to the same denaturation condition or a denaturing agent.

~~[0349]~~(246) For example, each member of a library or collection of modified hyaluronan-degrading enzymes is incubated under or exposed to one or more denaturation conditions. The incubation or exposure can occur in vivo or in vitro. Typically, the assay is performed in vitro. The same modified enzyme also is exposed or incubated to a reference or control condition that does not contain the denaturation condition. The activities under both conditions are compared in order to identify modified hyaluronan-degrading enzymes that exhibit stability upon exposure to a denaturation condition or conditions. Further, in screening or identifying the activity of the enzyme under the two different sets of conditions, generally the only conditions that are varied in the assay relate to the presence or absence of one or more denaturation conditions. The other

conditions of the assay, including but not limited to, time, temperature and/or other incubation conditions, can be the same for both sets of conditions.

[0350](247) For example, exposure can be achieved by incubation of a modified hyaluronan-degrading enzyme in an assay buffer or composition that has been modified or adjusted to contain a denaturing agent such as an excipient or low or no salt. Exemplary denaturing agents or excipients include, but are not limited to, antiadherents, binders, coatings, fillers and diluents, flavors, colors, lubricants, glidants, preservatives, sorbents or sweeteners. The choice of buffer that is used can be empirically determined by one skilled in the art depending on the particular parameter or parameters being modified. Exemplary assay buffers are Good's buffers (see e.g., Good et al. (1966) Biochemistry, 5:467-477). Examples of such buffers include, but are not limited to ACES, ADA, BES, Bicine, BIS-TRIS, CAPS, HEPES, MES, MOPS, PIPES, TRIS or Trizma.RTM.® buffers. Further, the amount or concentration of the excipient or salt can be empirically determined by one of skill in the art depending on the choice of excipient or salt and the desired level or activity of the modified hyaluronan-degrading enzyme.

[0351](248) In one example, the assay buffer or composition is modified by inclusion of an amount of a denaturing agent or denaturing excipient that is a preservative, for example;, a phenolic preservative. The phenolic preservative can be phenol, metacresol (m-cresol), benzyl alcohol, and parabens including methylparaben and propylparaben. In particular, the phenolic preservative is phenol and/or m-cresol. The total amount of one or more phenolic preservative agents as a percentage (%) of mass concentration (w/v) can be between 0.05% to 0.6%, 0.1% to 0.4%, 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3% or 0.3% to 0.4% inclusive. In such an example, the activity of the modified hyaluronan-degrading enzyme is tested or assessed in the presence of such a total amount (e.g., between or about between 0.05% to 0.6%) of one or more preservatives, for example, one or more phenolic preservatives. In some examples, the modified hyaluronan-degrading enzyme also can be tested or assessed under a control or reference condition in which the assay buffer or composition is not modified to contain a preservative. In certain instances, as a control, the activity of modified hyaluronan-degrading enzymes also can be compared to the corresponding unmodified hyaluronan-degrading enzyme not containing the modification(s) under conditions that contain a preservative agent and/or under conditions that do not contain a preservative agent.

[0352](249) In another example, the assay buffer is modified by the presence of a denaturation condition that is low or no salt. As discussed elsewhere herein, hyaluronan-degrading enzymes, such as PH20, generally require salt (e.g., NaCl, Lys-Lys or MgCl.sub.2) for activity. Hence, the absence of salt or low salt is denaturing to the enzyme. In one example, the assay buffer is modified by inclusion of an amount of salt that is less than 100 mM, for example, less than 90 mM, 80 mM, 70 mM, 60 mM, 50 mM, 40 mM, 30 mM, 25 mM, 20 mM, 15 mM, 10 mM, 5 mM or less. In such an example, the activity of the modified hyaluronan-degrading enzyme is tested in the absence of salt or in the presence of salt that is less than 100 mM. In some examples, the modified hyaluronan-degrading enzyme also can be tested or assessed under a control or reference condition in which the assay buffer contains a higher salt concentration, generally between or about between 140 mM to 200 mM. In certain instances, as a control, the activity of modified hyaluronan-degrading enzymes also can be compared to the corresponding unmodified hyaluronan-degrading enzyme not containing the modification(s) under conditions that contain

low or no salt, such as less than 100 mM and/or under conditions that contain salt in an amount that is between or about between 140 mM to 200 mM.

~~[0353]~~(250) Exposure of a hyaluronan-degrading enzyme to a denaturation condition also can be achieved by incubation of a modified ~~hyaluronan-degrading~~hyaluronan-de grading enzyme under conditions that are known to be denaturing, such as under conditions of elevated temperature such as a temperature greater than or about or 30~~.degree.~~° C. (e.g., 30~~.degree.~~° C. to 42~~.degree.~~° C. such as or about 37~~.degree.~~° C.) or agitation. For example, the activity of the modified hyaluronan-degrading enzyme is tested at elevated temperatures greater than or about or 30~~.degree.~~° C. to 42~~.degree.~~° C. In some examples, the modified hyaluronan-degrading enzyme also can be tested or assessed under a control or reference condition where the temperatures is less than 30~~.degree.~~° C., such as between or about between 0~~.degree.~~° C. to 25~~.degree.~~° C., for example, 0~~.degree.~~° C. to 5~~.degree.~~° C. or 18~~.degree.~~° C. to 25~~.degree.~~° C. In certain instances, as a control, the activity of modified hyaluronan-degrading enzymes also can be compared to the corresponding unmodified hyaluronan-degrading enzyme not containing the modification(s) under elevated temperatures greater than or about or 30~~.degree.~~° C. to 42~~.degree.~~° C. and/or temperatures is less than 30~~.degree.~~° C., such as between or about between 0~~.degree.~~° C. to 25~~.degree.~~° C., for example, 0~~.degree.~~° C. to 5~~.degree.~~° C. or 18~~.degree.~~° C. to 25~~.degree.~~° C.

~~[0354]~~(251) The modified hyaluronan-degrading enzyme can be exposed to one or more than one of the conditions. The exposure to one condition can occur simultaneously, subsequently, intermittently or periodically to exposure to one or more other conditions.

~~[0355]~~(252) In one example, in the method herein, the modified hyaluronan-degrading enzyme is incubated or exposed to the denaturation condition or denaturing agent prior to performing an assay for hyaluronidase activity. For example, the modified hyaluronan-degrading enzyme is incubated in the presence of a denaturing agent or exposed to one or more denaturation conditions or control conditions, such as one or more of the denaturation conditions or control conditions as described above. The incubation or exposure can be for any desired length of time, and can be empirically determined by one of skill in the art. For example, the modified hyaluronan-degrading enzyme can be incubated or exposed to one or more denaturation conditions, denaturing agents or control conditions for or about for 1 minute to 1 month, such as 1 minute to 3 weeks, 1 minute to 2 weeks, 1 minute to 1 week, 1 minute to 24 hours, 1 minute to 12 hours, such as 30 minutes to 6 hours or 1 hour to 4 hours, and generally at least or about at least 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours or 12 hours. After the time of incubation or exposure, the sample or composition containing the modified hyaluronan-degrading enzyme (or control unmodified enzyme) is assessed for hyaluronidase assay. In another example, the modified hyaluronan-degrading enzyme is exposed or incubated under one or more denaturation conditions and is simultaneously or concurrently assessed for hyaluronidase activity. In any examples where a modified hyaluronan-degrading enzyme is assessed, it is understood that an unmodified hyaluronan-degrading enzyme not containing the modifications(s) also can be assessed under similar assay conditions for comparison.

~~[0356]~~(253) Assays to assess hyaluronidase activity are well known in the art. Examples of such assays are described in Section G. In one example, hyaluronidase activity can be assessed in a microturbidity assay, wherein the amount of undegraded HA is measured by the addition of a

reagent that precipitates HA (e.g., Cetylpyridinium chloride (CPC) or acidified serum) after the enzyme is allowed to react with HA. In another example, hyaluronidase activity can be assessed using a microtiter assay in which residual biotinylated hyaluronic acid is measured following incubation with hyaluronidase (see e.g., Frost and Stern (~~997~~1997) Anal. Biochem. 251:263-269, U.S. Pat. Publication No. 20050260186). The resulting activities under each of the tested conditions is determined and compared.

~~[0357]~~(254) 3. Selection or Identification

~~[0358]~~(255) In the method, after screening modified hyaluronan-degrading enzymes under one or more denaturation conditions, the hyaluronidase activities of the tested enzymes are compared. The method is practiced in order to identify a modified hyaluronan-degrading enzyme that is more resistant to denaturation by a condition or a denaturing agent, whereby the activity of the enzyme is indicative of the stability of the enzyme as a measure of its resistance to denaturation. It is understood that some reduction of enzyme activity, as a result of denaturation, can be tolerated in various applications, and thus the method can be practiced to select for a modified hyaluronan-degrading enzymes that exhibits a requisite activity upon exposure to a denaturation condition to permit its use or application (e.g., therapeutic activity). For example, a modified enzyme can be selected that loses activity more slowly than the corresponding unmodified or reference hyaluronan-degrading enzyme, but whose retained activity is sufficient for a particular application or purpose.

~~[0359]~~(256) In examples of the methods herein, the activity of the modified hyaluronan degrading enzyme is assessed upon exposure to a first denaturation condition and also assessed upon exposure to a second condition that is a control or non-denaturation condition, and the resulting hyaluronidase activities compared. For comparison, in some examples, the activity can be represented as a ratio of activity or a percentage of activity under a denaturation condition compared to under a control or non-denaturation condition. For example, where the parameter that differs between the first and second condition is the presence of preservative (e.g., phenolic preservative), activity can be represented as a ratio of activity or percentage of activity observed in the presence of preservative (e.g., phenolic preservative) versus activity in the absence of preservative (e.g., phenolic preservative). In another example, where the parameter that differs between the first and second condition is temperature, activity can be represented as a ratio of activity or percentage of activity observed in the presence of elevated temperature (e.g., 30~~.degree.~~° C. to 42~~.degree.~~° C.) compared to activity in the presence of a lower temperature such as 0~~.degree.~~° C. to 25~~.degree.~~° C., for example 0~~.degree.~~° C. to 5~~.degree.~~° C. or 18~~.degree.~~° C. to 25~~.degree.~~° C.

~~[0360]~~(257) A modified hyaluronan-degrading enzyme is selected or identified that retains or exhibits any desired activity in the presence of the denaturation condition compared to in its absence. The particular cut-off of activity for selection of enzymes herein is dependent on the particular user and/or practice of the method and can be empirically determined depending on factors such as the particular denaturation condition or denaturing agent, the particular modified hyaluronan-degrading enzyme, the desired application of the identified or selected hyaluronan-degrading enzyme and other similar factors. Generally, a selected or identified modified hyaluronan-degrading enzyme exhibits stability if any detectable activity is measured or assessed upon exposure or incubation with a denaturation condition or denaturing agent. For example, a

selected or identified modified hyaluronan-degrading enzyme exhibits stability, or resistance to a denaturation condition or denaturing agent, if it exhibits at least 5% or 10% of the activity of the same enzyme in the absence of the denaturation condition or denaturing agent, and generally if the modified hyaluronan-degrading enzyme exhibits an activity that is at least 15% of the initial hyaluronidase activity prior to incubation in the presence of the denaturation condition. For example, a modified hyaluronan-degrading enzyme is selected or identified that exhibits at least (or at least about) 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, 200%, 300%, 400%, 500% or more of the initial hyaluronidase activity of the modified ~~hyaluronan-degrading~~hyaluronan-de grading enzyme tested under a control or non-denaturation condition.

~~[0361]~~(258) In other examples of the methods herein, the activity of the modified hyaluronan degrading enzyme is assessed upon exposure to a denaturation condition and the activity of the unmodified or reference hyaluronan-degrading enzyme also is assessed upon exposure to the same denaturation conditions. In such examples, the activities are compared when the enzymes are exposed to the same conditions. For comparison, the activity under a denaturation condition can be represented as a ratio of activity or a percentage of activity of a modified hyaluronan-degrading enzyme compared to an unmodified or reference hyaluronan-degrading enzyme. In such examples, a modified hyaluronan-degrading enzyme is selected that exhibits greater activity under a denaturation condition than the unmodified or reference hyaluronan-degrading enzyme. Thus, the modified hyaluronan-degrading enzyme is one that is more resistant to the condition. For example, where the denaturation condition is the presence of preservative (e.g., phenolic preservative), the activity observed in the presence of preservative (e.g., phenolic preservative) can be represented as a ratio of activity or percentage of activity of the modified hyaluronan-degrading enzyme compared to the unmodified or reference hyaluronan-degrading enzyme. In another example, where the denaturation condition is high temperature, activity observed in the presence of elevated temperature (e.g., 30~~.degree.~~° C. to 42~~.degree.~~° C.) can be represented as a ratio of activity or percentage of activity of the modified hyaluronan-degrading enzyme compared to the unmodified or reference hyaluronan-degrading enzyme.

~~[0362]~~(259) In such examples, a modified hyaluronan-degrading enzyme, such as a modified PH20, is identified or selected that exhibits a ratio of activity that is greater than or at least 1.1, such that the enzyme exhibits greater activity than the unmodified or reference hyaluronan-degrading enzyme under the denaturation condition. For example, the ratio is at least or at least about 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 or greater. A modified hyaluronan-degrading enzyme (e.g., a modified PH20) can be selected if its activity is at least 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 250%, 300%, 400%, 500% or more of the activity of the unmodified or reference hyaluronan-degrading enzyme when tested under the same conditions. Thus, modified hyaluronan-degrading enzymes are identified that exhibit greater or improved stability compared to the unmodified hyaluronan-degrading enzyme or a reference hyaluronan-degrading enzyme as manifested by increased resistance to a denaturation condition or denaturing agent.

~~[0363]~~(260) 4. Iterative Methods

~~[0364]~~(261) The method provided herein also is iterative. In one example, after the method is performed, any modified hyaluronan-degrading enzymes identified as exhibiting stability, such

as increased stability, under a denaturation condition can be modified or further modified to increase or optimize the stability. A secondary library can be created by introducing additional modifications in a first identified modified hyaluronan-degrading enzyme. For example, modifications that were identified as conferring stability, such as increasing stability, can be combined to generate a combinatorial library. The secondary library can be tested using the assays and methods described herein.

~~[0365]~~(262) In another example of an iterative aspect of the method, modified hyaluronan-degrading enzymes that are identified as not exhibiting stability such as increased stability (e.g., such that they are not active or do not have increased activity under the a denaturation condition), can be further modified and retested for stability under a denaturation condition. The further modifications can be targeted near particular regions (e.g., particular amino acid residues) associated with activity and/or stability of the molecule. For example, residues that are associated with activity and/or stability of the molecule generally are critical residues that are involved in the structural folding or other activities of the molecule. Hence, such residues are required for activity, generally under any condition. Critical residues can be identified because, when mutated, a normal activity of the protein is ablated or reduced. For example, critical residues can be identified that, when mutated in a hyaluronan-degrading enzyme, exhibit reduced or ablated hyaluronidase activity under a normal or control assay condition. A further library of modified proteins can be generated with amino acid mutations targeted at or near to the identified critical amino acid residues, such as adjacent to the identified critical amino acid residues. In some examples, the mutations can be amino acid replacement to any other of up to 19 other amino acid residues. The secondary library can be tested using the assays and methods described herein.

E. ~~Production of Modified PH20 Polypeptides and Encoding Nucleic Acid Molecules~~PRODUCTION OF MODIFIED PH20 POLYPEPTIDES AND ENCODING NUCLEIC ACID MOLECULES

~~[0366]~~(263) Polypeptides of a modified PH20 polypeptide set forth herein can be obtained by methods well known in the art for protein purification and recombinant protein expression. Polypeptides also can be synthesized chemically. Modified or variant, including truncated, forms can be engineered from a wildtype polypeptide using standard recombinant DNA methods. For example, modified PH20 polypeptides can be engineered from a wildtype polypeptide, such as by site-directed mutagenesis.

~~[0367]~~(264) 1. Isolation or Preparation of Nucleic Acids Encoding PH20 Polypeptides

~~[0368]~~(265) Polypeptides can be cloned or isolated using any available methods known in the art for cloning and isolating nucleic acid molecules. Such methods include PCR amplification of nucleic acids and screening of libraries, including nucleic acid hybridization screening, antibody-based screening and activity-based screening.

~~[0369]~~(266) For example, when the polypeptides are produced by recombinant means, any method known to those of skill in the art for identification of nucleic acids that encode desired genes can be used. Any method available in the art can be used to obtain a full length or partial

(i.e., encompassing the entire coding region) cDNA or genomic DNA clone encoding a PH20, such as from a cell or tissue source.

[0370](267) Methods for amplification of nucleic acids can be used to isolate nucleic acid molecules encoding a desired polypeptide, including for example, polymerase chain reaction (PCR) methods. Examples of such methods include use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp). A nucleic acid containing material can be used as a starting material from which a desired polypeptide-encoding nucleic acid molecule can be isolated. For example, DNA and mRNA preparations, cell extracts, tissue extracts, fluid samples (e.g., blood, serum, saliva), samples from healthy and/or diseased subjects can be used in amplification methods. The source can be from any eukaryotic species including, but not limited to, vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, and other primate sources. Nucleic acid libraries also can be used as a source of starting material. Primers can be designed to amplify a desired polypeptide. For example, primers can be designed based on expressed sequences from which a desired polypeptide is generated. Primers can be designed based on back-translation of a polypeptide amino acid sequence. If desired, degenerate primers can be used for amplification. Oligonucleotide primers that hybridize to sequences at the 3′ and 5′ termini of the desired sequence can be uses as primers to amplify by PCR sequences from a nucleic acid sample. Primers can be used to amplify the entire full-length PH20, or a truncated sequence thereof, such as a nucleic acid encoding any of the soluble PH20 polypeptides provided herein. Nucleic acid molecules generated by amplification can be sequenced and confirmed to encode a desired polypeptide.

[0371](268) Additional nucleotide sequences can be joined to a polypeptide-encoding nucleic acid molecule, including linker sequences containing restriction endonuclease sites for the purpose of cloning the synthetic gene into a vector, for example, a protein expression vector or a vector designed for the amplification of the core protein coding DNA sequences. Furthermore, additional nucleotide sequences specifying functional DNA elements can be operatively linked to a polypeptide-encoding nucleic acid molecule. Examples of such sequences include, but are not limited to, promoter sequences designed to facilitate intracellular protein expression, and secretion sequences, for example heterologous signal sequences, designed to facilitate protein secretion. Such sequences are known to those of skill in the art. For example, exemplary heterologous signal sequences include, but are not limited to, human and mouse kappa IgG heterologous signal sequences set forth in SEQ ID NO: 868. Additional nucleotide residue sequences such as sequences of bases specifying protein binding regions also can be linked to enzyme-encoding nucleic acid molecules. Such regions include, but are not limited to, sequences of residues that facilitate or encode proteins that facilitate uptake of an enzyme into specific target cells, or otherwise alter pharmacokinetics of a product of a synthetic gene.

[0372](269) In addition, tags or other moieties can be added, for example, to aid in detection or affinity purification of the polypeptide. For example, additional nucleotide residue sequences such as sequences of bases specifying an epitope tag or other detectable marker also can be linked to enzyme-encoding nucleic acid molecules. Examples of such sequences include nucleic acid sequences encoding a His tag or Flag Tag.

[0373](270) The identified and isolated nucleic acids can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art can be used. Possible

vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pCMV4, pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene, La Jolla, Calif.). Other expression vectors include the HZ24 expression vector exemplified herein (see e.g., SEQ ID NOS:4 and 5). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. Insertion can be effected using TOPO cloning vectors (Invitrogen, Carlsbad, Calif.).

~~[0374]~~(271) If the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules can be enzymatically modified. Alternatively, any site desired can be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers can contain specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and protein gene can be modified by homopolymeric tailing.

~~[0375]~~(272) Recombinant molecules can be introduced into host cells via, for example, transformation, transfection, infection, electroporation and sonoporation, so that many copies of the gene sequence are generated. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated protein gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene can be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

~~[0376]~~(273) In addition to recombinant production, modified PH20 polypeptides provided herein can be produced by direct peptide synthesis using solid-phase techniques (see e.g., Stewart et al. (1969) Solid-Phase Peptide Synthesis, WH Freeman Co., San Francisco; Merrifield J (~~963~~1963) J Am Chem Soc., 85:2149-2154). In vitro protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City Calif.) in accordance with the instructions provided by the manufacturer. Various fragments of a polypeptide can be chemically synthesized separately and combined using chemical methods.

~~[0377]~~(274) 2. Generation of Mutant ~~of~~or Modified Nucleic Acid and Encoding Polypeptides

~~[0378]~~(275) The modifications provided herein can be made by standard recombinant DNA techniques such as are routine to one of skill in the art. Any method known in the art to effect mutation of any one or more amino acids in a target protein can be employed. Methods include standard site-directed mutagenesis (using e.g., a kit, such as QuikChange available from Stratagene) of encoding nucleic acid molecules, or by solid phase polypeptide synthesis methods.

~~[0379]~~(276) 3. Vectors and Cells

~~[0380]~~(277) For recombinant expression of one or more of the desired proteins, such as any modified PH20 polypeptide described herein, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can be inserted into an appropriate expression vector,

i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence. The necessary transcriptional and translational signals also can be supplied by the native promoter for enzyme genes, and/or their flanking regions.

~~[0381]~~(278) Also provided are vectors that contain a nucleic acid encoding the enzyme. Cells containing the vectors also are provided. The cells include eukaryotic and prokaryotic cells, and the vectors are any suitable for use therein. Generally, the cell is a cell that is capable of effecting ~~glyosylation~~glycosylation of the encoded protein.

~~[0382]~~(279) Prokaryotic and eukaryotic cells containing the vectors are provided. Such cells include bacterial cells, yeast cells, fungal cells, Archea, plant cells, insect cells and animal cells. The cells are used to produce a protein thereof by growing the above-described cells under conditions whereby the encoded protein is expressed by the cell, and recovering the expressed protein. For purposes herein, for example, the enzyme can be secreted into the medium.

~~[0383]~~(280) A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing can impact the folding and/or function of the polypeptide. Different host cells, such as, but not limited to, CHO (DG44, DXB11, CHO-K1), HeLa, MCDK, 293 and WI38 have specific cellular machinery and characteristic mechanisms for such post-translational activities and can be chosen to ensure the correct modification and processing of the introduced protein. Generally, the choice of cell is one that is capable of introducing N-linked glycosylation into the expressed polypeptide. Hence, eukaryotic cells containing the vectors are provided. Exemplary eukaryotic cells are mammalian Chinese Hamster Ovary (CHO) cells. For example, CHO cells deficient in dihydrofolate reductase (e.g., DG44 cells) are used to produce polypeptides provided herein. Note that bacterial expression of an PH20 polypeptide provided herein will not result in a catalytically active polypeptide, but when combined with proper glycosylation machinery, the PH20 can be artificially glycosylated.

~~[0384]~~(281) Provided are vectors that contain a sequence of nucleotides that encodes the modified PH20 polypeptide, coupled to the native or heterologous signal sequence, as well as multiple copies thereof. The vectors can be selected for expression of the enzyme protein in the cell or such that the enzyme protein is expressed as a secreted protein.

~~[0385]~~(282) A variety of host-vector systems can be used to express the protein encoding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus and other viruses); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system used, any one of a number of suitable transcription and translation elements can be used.

~~[0386]~~(283) Any methods known to those of skill in the art for the insertion of DNA fragments into a vector can be used to construct expression vectors containing a chimeric gene containing appropriate transcriptional/translational control signals and protein coding sequences. These

methods can include in vitro recombinant DNA and synthetic techniques and in vivo recombinants (genetic recombination). Expression of nucleic acid sequences encoding protein, or domains, derivatives, fragments or homologs thereof, can be regulated by a second nucleic acid sequence so that the genes or fragments thereof are expressed in a host transformed with the recombinant DNA molecule(s). For example, expression of the proteins can be controlled by any promoter/enhancer known in the art. In a specific embodiment, the promoter is not native to the genes for a desired protein. Promoters which can be used include, but are not limited to, the SV40 early promoter (Bernoist and Chambon, Nature 290:304-310 (1981)), the promoter contained in the 3'' long terminal repeat of Rous sarcoma virus (Yamamoto et al. Cell 22:787-797 (1980)), the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. USA 78:1441-1445 (1981)), the regulatory sequences of the metallothionein gene (Brinster et al., Nature 296:39-42 (1982)); prokaryotic expression vector promoters, such as the ~~.beta.-lactamase~~β-lactamase promoter (Jay et al., (1981) Proc. Natl. Acad. Sci. USA ~~75~~78:5543) or the tac promoter (DeBoer et al., Proc. Natl. Acad. Sci. USA 80:21-25 (1983); see also Gilbert and Villa-Komaroff, ~~"~~"Useful Proteins from Recombinant Bacteria,~~"~~" Scientific American 242:74-94 (1980)); plant expression vector promoters, such as the nopaline synthetase promoter (Herrera-Estrella et al., Nature ~~305~~303:209-213 (1984)) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., Nucleic Acids Res. 9:2871 (1981)), and the promoter of the photosynthetic enzyme ribulose bisphosphate carboxylase (Herrera-Estrella et al., Nature 310:115-120 (1984)); promoter elements from yeast and other fungi such as the Gal4 promoter, the alcohol dehydrogenase promoter, the phosphoglycerol kinase promoter, the alkaline phosphatase promoter, and the following animal transcriptional control regions that exhibit tissue specificity and have been used in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., Cell ~~55~~38:639-646 (1984); Ornitz et al., Cold Spring Harbor Symp. Quant. Biol. 50:399-409 (1986); MacDonald, Hepatology 7:425-515 (1987)); insulin gene control region which is active in pancreatic beta cells (Hanahan et al., Nature ~~375~~315:115-122 (1985)), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., Cell ~~55~~38:647-658 (1984); Adams et al., Nature ~~575~~318:533-538 (1985); Alexander et al., Mol. Cell~~.~~ Biol. 7:1436-1444 (1987)), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., Cell 45:485-495 (1986)), albumin gene control region which is active in liver (Pinkert et al., Genes and Devel. ~~7~~1:268-276 (1987)), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., Mol. Cell. Biol. 5:1639-1648 (1985); Hammer et al., Science ~~255~~235:53-58 1987)), alpha-1 antitrypsin gene control region which is active in liver (Kelsey et al., Genes and Devel. ~~7~~1:161-171 (1987)), beta globin gene control region which is active in myeloid cells (Magram et al., Nature ~~575~~315:338-340 (1985); Kollias et al., Cell 46:89-94 (1986)), myelin basic protein gene control region which is active in oligodendrocyte cells of the brain (Readhead et al., Cell ~~45~~48:703-712 (1987)), myosin light chain-2 gene control region which is active in skeletal muscle (Shani, Nature ~~574~~314:283-286 (1985)), and gonadotrophic releasing hormone gene control region which is active in gonadotrophs of the hypothalamus (Mason et al., Science 234:1372-1378 (1986)).

~~[0387]~~(284) In a specific embodiment, a vector is used that contains a promoter operably linked to nucleic acids encoding a desired protein, or a domain, fragment, derivative or homolog thereof, one or more origins of replication, and optionally, one or more selectable markers (e.g., an antibiotic resistance gene). Depending on the expression system, specific initiation signals also are required for efficient translation of a PH20 sequence. These signals include the ATG

initiation codon and adjacent sequences. In cases where the initiation codon and upstream sequences of PH20 or soluble forms thereof are inserted into the appropriate expression vector, no additional translational control signals are needed. In cases where only a coding sequence, or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf et al. (1994) Results Probl Cell Differ 20:125-62; Bittner et al. (1987) Methods in Enzymol, 153:516-544).

[0388](285) Exemplary plasmid vectors for transformation of E. coli cells include, for example, the pQE expression vectors (available from Qiagen, Valencia, Calif.; see also literature published by Qiagen describing the system). pQE vectors have a phage T5 promoter (recognized by E. coli RNA polymerase) and a double lac operator repression module to provide tightly regulated, high-level expression of recombinant proteins in E. coli, a synthetic ribosomal binding site (RBS II) for efficient translation, a 6.times.×His tag coding sequence, tot.sub.0 and T1 transcriptional terminators, ColE1 origin of replication, and a beta-lactamase gene for conferring ampicillin resistance. The pQE vectors enable placement of a 6.times.×His tag at either the N- or C-terminus of the recombinant protein. Such plasmids include pQE 32, pQE 30, and pQE 31 which provide multiple cloning sites for all three reading frames and provide for the expression of N-terminally 6.times.×His-tagged proteins. Other exemplary plasmid vectors for transformation of E. coli cells, include, for example, the pET expression vectors (see, U.S. Pat. No. 4,952,496; available from Novagen, Madison, Wis.; see, also literature published by Novagen describing the system). Such plasmids include pET 11apET11a, which contains the T7lac promoter, T7 terminator, the inducible E. coli lac operator, and the lac repressor gene; pET 12a-c, which contains the T7 promoter, T7 terminator, and the E. coli ompT secretion signal; and pET 15bpET15b and pET19b (Novagen, Madison, Wis.), which contain a His-Tag.TM.™ leader sequence for use in purification with a H isHis column and a thrombin cleavage site that permits cleavage following purification over the column, the T7-lac promoter region and the T7 terminator.

[0389](286) Typically, vectors can be plasmids, viral vectors, or others known in the art, used for expression of the modified PH20 polypeptide in vivo or in vitro. For example, the modified PH20 polypeptide is expressed in mammalian cells, including, for example, Chinese Hamster

[0390] Ovary (CHO) cells. An exemplary vector for mammalian cell expression is the HZ24 expression vector. The HZ24 expression vector was derived from the pCI vector backbone[0391] (Promega). It contains DNA encoding the Beta-lactamase resistance gene (AmpR), an F1 origin of replication, a Cytomegalovirus immediate-early enhancer/promoter region (CMV), and an SV40 late polyadenylation signal (SV40). The expression vector also has an internal ribosome entry site (ERESIRES) from the ECMV virus (Clontech) and the mouse dihydrofolate reductase (DHFR) gene.

[0392](287) Viral vectors, such as adenovirus, retrovirus or vaccinia virus vectors, can be employed. In some examples, the vector is a defective or attenuated retroviral or other viral vector (see U.S. Pat. No. 4,980,286). For example, a retroviral vector can be used (see Miller et

al., Meth. Enzymol. 217: 581-599 (1993)). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA.

[0393](288) In some examples, viruses armed with a nucleic acid encoding a modified PH20 polypeptide can facilitate their replication and spread within a target tissue for example. The target tissue can be a cancerous tissue whereby the virus is capable of selective replication within the tumor. The virus can also be a non-lytic virus wherein the virus selectively replicates under a tissue specific promoter. As the viruses replicate, the coexpression of the PH20 polypeptide with viral genes will facilitate the spread of the virus in vivo.

[0394](289) 4. Expression

[0395](290) Modified PH20 polypeptides can be produced by any method known to those of skill in the art including in vivo and in vitro methods. Desired proteins can be expressed in any organism suitable to produce the required amounts and forms of the proteins, such as for example, those needed for administration and treatment. Expression hosts include prokaryotic and eukaryotic organisms such as E. coli, yeast, plants, insect cells, mammalian cells, including human cell lines and transgenic animals. Expression hosts can differ in their protein production levels as well as the types of post-translational modifications that are present on the expressed proteins. The choice of expression host can be made based on these and other factors, such as regulatory and safety considerations, production costs and the need and methods for purification.

[0396](291) Many expression vectors are available and known to those of skill in the art and can be used for expression of proteins. The choice of expression vector will be influenced by the choice of host expression system. In general, expression vectors can include transcriptional promoters and optionally enhancers, translational signals, and transcriptional and translational termination signals. Expression vectors that are used for stable transformation typically have a selectable marker which allows selection and maintenance of the transformed cells. In some cases, an origin of replication can be used to amplify the copy number of the vector.

[0397](292) Modified PH20 polypeptides also can be utilized or expressed as protein fusions. For example, an enzyme fusion can be generated to add additional functionality to an enzyme. Examples of enzyme fusion proteins include, but are not limited to, fusions of a signal sequence, a tag such as for localization, e.g., a 6.times.×His or His.sub.6 tag or a myc tag, or a tag for purification, for example, a GST fusion, and a sequence for directing protein secretion and/or membrane association.

[0398](293) For long-term, high-yield production of recombinant proteins, stable expression is desired. For example, cell lines that stably express a modified PH20 polypeptide can be transformed using expression vectors that contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells that successfully express the introduced sequences. Resistant cells of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell types.

[0399](294) Any number of selection systems can be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M et al. (1977) Cell, 11:223-32) and adenine phosphoribosyltransferase (Lowy, I et al. (1980) Cell, 22:817-23) genes, which can be employed in TK- or APRT- cellsAPRT-cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection. For example, DHFR, which confers resistance to methotrexate (Wigler, M et al. (1980) Proc. Natl. Acad. Sci, 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F et al. (1981) J. Mol. Biol., 150:1-14); and als or pat, which confer resistance to chlorsulfuron and phosphinotricinphosphinothricin acetyltransferase, respectively, can be used. Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of typtophantryptophan or hisD, which allows cells to utilize histinol in place of histidine (Hartman S C and R C Mulligan (1988) Proc. Natl. Acad. Sci, 85:8047-51). Visible markers, such as but not limited to, anthocyanins, beta glucuronidase and its substrate, GUS, and luciferase and its substrate luciferin, also can be used to identify transformants and also to quantify the amount of transient or stable protein expression attributable to a particular vector system (Rhodes C A et al. (1995) Methods Mol. Biol. 55:121-131).

[0400](295) The presence and expression of PH20 polypeptides can be monitored. For example, detection of a functional polypeptide can be determined by testing the conditioned media for hyaluronidase enzyme activity under appropriate conditions. Exemplary assays to assess the solubility and activity of expressed proteins are provided herein.

[0401](296) a. Prokaryotic Cells

[0402](297) Prokaryotes, especially E. coli, provide a system for producing large amounts of proteins. Transformation of E. coli is a simple and rapid technique well known to those of skill in the art. Expression vectors for E. coli can contain inducible promoters. Such promoters are useful for inducing high levels of protein expression and for expressing proteins that exhibit some toxicity to the host cells. Examples of inducible promoters include the lac promoter, the trp promoter, the hybrid tac promoter, the T7 and SP6 RNA promoters and the temperature regulated .lamda.PLλPL promoter.

[0403](298) Proteins, such as any provided herein, can be expressed in the cytoplasmic environment of E. coli. The cytoplasm is a reducing environment, and for some molecules, this can result in the formation of insoluble inclusion bodies. Reducing agents such as dithiothreotol and .beta.- mercaptoethanolβ-mercaptoethanol and denaturants, such as guanidine-HCl and urea can be used to resolubilize the proteins. An alternative approach effects protein expression in the periplasmic space of bacteria which provides an oxidizing environment and chaperonin-like and disulfide isomerases, which can aid in the production of soluble protein. Typically, a leader sequence is fused to the protein to be expressed which directs the protein to the periplasm. The leader is then removed by signal peptidases inside the periplasm. Examples of periplasmic-targeting leader sequences include the pelB leader from the pectate lyase gene and the leader derived from the alkaline phosphatase gene. In some cases, periplasmic expression allows leakage of the expressed protein into the culture medium. The secretion of proteins allows quick and simple purification from the culture supernatant. Proteins that are not secreted can be obtained from the periplasm by osmotic lysis. Similar to cytoplasmic expression, in some cases

proteins can become insoluble and denaturants and reducing agents can be used to facilitate solubilization and refolding. Temperature of induction and growth ~~1~~ also can influence expression levels and solubility, typically temperatures between 25~~.degree.~~° C. and 37~~.degree.~~° C. are used. Typically, bacteria produce aglycosylated proteins. Thus, if proteins require glycosylation for function, glycosylation can be added in vitro after purification from host cells.

~~[0404]~~(299) b. Yeast Cells

~~[0405]~~(300) Yeasts such as Saccharomyces cerevisae, Schizosaccharomyces pombe, Yarrowia lipolytica, Kluyveromyces lactis and Pichia pastoris are well known yeast expression hosts that can be used for production of proteins, such as any described herein. Yeast can be transformed with episomal replicating vectors or by stable chromosomal integration by homologous recombination. Typically, inducible promoters are used to regulate gene expression. Examples of such promoters include GAL1, GAL7 and GAL5 and metallothionein promoters, such as CUP1, AOX1 or other Pichia or other yeast promoters. Expression vectors often include a selectable marker such as LEU2, TRP1, HIS3 and URA3 for selection and maintenance of the transformed DNA. Proteins expressed in yeast are often soluble. Co-expression with chaperonins such as Bip and protein disulfide isomerase can improve expression levels and solubility. Additionally, proteins expressed in yeast can be directed for secretion using secretion signal peptide fusions such as the yeast mating type alpha-factor secretion signal from Saccharomyces cerevisae and fusions with yeast cell surface proteins such as the Aga2p mating adhesion receptor or the Arxula adeninivorans glucoamylase. A protease cleavage site such as for the Kex-2 protease, can be engineered to remove the fused sequences from the expressed polypeptides as they exit the secretion pathway. Yeast also is capable of glycosylation at Asn-X-Ser/Thr motifs.

~~[0406]~~(301) c. Insects and Insect Cells

~~[0407]~~(302) Insect cells, particularly using baculovirus expression, are useful for expressing polypeptides such as PH20 polypeptides. Insect cells express high levels of protein and are capable of most of the post-translational modifications used by higher eukaryotes. Baculoviruses have a restrictive host range which improves the safety and reduces regulatory concerns of eukaryotic expression. Typical expression vectors use a promoter for high level expression such as the polyhedrin promoter of baculovirus. Commonly used baculovirus systems include a baculovirus, such as the Autographa californica nuclear polyhedrosis virus (AcNPV) or the ~~bombyx~~Bombyx mori nuclear polyhedrosis virus (BmNPV), and an insect cell line, such as Sf9 derived from Spodoptera frugiperda, Pseudaletia unipuncta (A7S) and Danaus plexippus (DpN1). For high-level expression, the nucleotide sequence of the molecule to be expressed is fused immediately downstream of the polyhedrin initiation codon of the virus. Mammalian secretion signals are accurately processed in insect cells and can be used to secrete the expressed protein into the culture medium. In addition, the cell lines Pseudaletia unipuncta (A7S) and Danaus plexippus (DpN1) produce proteins with glycosylation patterns similar to mammalian cell systems. Exemplary insect cells are those that have been altered to reduce immunogenicity, including those with ~~"~~“mammalianized~~"~~” baculovirus expression vectors and those lacking the enzyme FT3.

~~[0408]~~(303) An alternative expression system in insect cells employs stably transformed cells. Cell lines such as the Schnieder 2 (S2) and Kc cells (Drosophila melanogaster) and C7 cells

(Aedes albopictus) can be used for expression. The Drosophila metallothionein promoter can be used to induce high levels of expression in the presence of heavy metal induction with cadmium or copper. Expression vectors are typically maintained by the use of selectable markers such as neomycin and hygromycin.

[0409](304) d. Mammalian Expression

[0410](305) Mammalian expression systems can be used to express proteins including PH20 polypeptides. Expression constructs can be transferred to mammalian cells by viral infection such as by adenovirus or by direct DNA transfer such as liposomes, calcium phosphate, DEAE-dextran and by physical means such as electroporation and microinjection. Expression vectors for mammalian cells typically include an mRNA cap site, a TATA box, a translational initiation sequence (Kozak consensus sequence) and polyadenylation elements. IRES elements also can be added to permit bicistronic expression with another gene, such as a selectable marker. Such vectors often include transcriptional promoter-enhancers for high-level expression, for example the SV40 promoter-enhancer, the human cytomegalovirus (CMV) promoter and the long terminal repeat of Rous sarcoma virus (RSV). These promoter-enhancers are active in many cell types. Tissue and cell-type promoters and enhancer regions also can be used for expression. Exemplary promoter/enhancer regions include, but are not limited to, those from genes such as elastase I, insulin, immunoglobulin, mouse mammary tumor virus, albumin, alpha fetoprotein, alpha 1 antitrypsin, beta globin, myelin basic protein, myosin light chain 2, and gonadotropic releasing hormone gene control. Selectable markers can be used to select for and maintain cells with the expression construct. Examples of selectable marker genes include, but are not limited to, hygromycin B phosphotransferase, adenosine deaminase, xanthine-guanine phosphoribosyl transferase, aminoglycoside phosphotransferase, dihydrofolate reductase (DHFR) and thymidine kinase. For example, expression can be performed in the presence of methotrexate to select for only those cells expressing the DHFR gene. Fusion with cell surface signaling molecules such as TCR-.zeta. andTCR-ξ nd Fc.sub..epsilon.RI-.gamma.εRI-γ can direct expression of the proteins in an active state on the cell surface.

[0411](306) Many cell lines are available for mammalian expression including mouse, rat human, monkey, chicken and hamster cells. Exemplary cell lines include but are not limited to CHO, Balb/3T3, HeLa, MT2, mouse NS0 (nonsecreting) and other myeloma cell lines, hybridoma and heterohybridoma cell lines, lymphocytes, fibroblasts, Sp2/0, COS, NIH3T3, HEK293, 293S, 2B8, and HKB cells. Cell lines also are available adapted to serum-free media which facilitates purification of secreted proteins from the cell culture media. Examples include CHO-SCHO—S cells (Invitrogen, Carlsbad, Calif., cat #11619-012) and the serum free EBNA-1 cell line (Pham et al., (2003) Biotechnol. Bioeng. 5484:332-42.). Cell lines also are available that are adapted to grow in special mediums optimized for maximal expression. For example, DG44 CHO cells are adapted to grow in suspension culture in a chemically defined, animal product-free medium.

[0412](307) e. Plants and Plant Cells

[0413](308) Transgenic plant cells and plants can be used to express proteins such as any described herein. Expression constructs are typically transferred to plants using direct DNA transfer such as microprojectile bombardment and PEG-mediated transfer into protoplasts, and

with agrobacterium-mediated transformation. Expression vectors can include promoter and enhancer sequences, transcriptional termination elements and translational control elements. Expression vectors and transformation techniques are usually divided between dicot hosts, such as Arabidopsis and tobacco, and monocot hosts, such as corn and rice. Examples of plant promoters used for expression include the cauliflower mosaic virus promoter, the nopaline ~~syntase~~synthase promoter, the ribose bisphosphate carboxylase promoter and the ubiquitin and UBQ3 promoters. Selectable markers such as hygromycin, phosphomannose isomerase and neomycin phosphotransferase are often used to facilitate selection and maintenance of transformed cells. Transformed plant cells can be maintained in culture as cells, aggregates (callus tissue) or regenerated into whole plants. Transgenic plant cells also can include algae engineered to produce hyaluronidase polypeptides. Because plants have different glycosylation patterns than mammalian cells, this can influence the choice of protein produced in these hosts.

~~[0414]~~(309) 5. Purification

~~[0415]~~(310) Host cells transformed with a nucleic acid sequence encoding a modified PH20 polypeptide can be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein produced by a recombinant cell is generally secreted, but may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing nucleic acid encoding PH20 can be designed with signal sequences that facilitate direct secretion of PH20 through prokaryotic or eukaryotic cell membranes.

~~[0416]~~(311) Thus, methods for purification of polypeptides from host cells will depend on the chosen host cells and expression systems. For secreted molecules, proteins are generally purified from the culture media after removing the cells. For intracellular expression, cells can be lysed and the proteins purified from the extract. When transgenic organisms such as transgenic plants and animals are used for expression, tissues or organs can be used as starting material to make a lysed cell extract. Additionally, transgenic animal production can include the production of polypeptides in milk or eggs, which can be collected, and if necessary, the proteins can be extracted and further purified using standard methods in the art.

~~[0417]~~(312) Proteins, such as modified PH20 polypeptides, can be purified using standard protein purification techniques known in the art including but not limited to, SDS-PAGE, size fractionation and size exclusion chromatography, ammonium sulfate precipitation and ionic exchange chromatography, such as anion exchange chromatography. Affinity purification techniques also can be utilized to improve the efficiency and purity of the preparations. For example, antibodies, receptors and other molecules that bind PH20 hyaluronidase enzymes can be used in affinity purification. For example, soluble PH20 can be purified from conditioned media.

~~[0418]~~(313) Expression constructs also can be engineered to add an affinity tag to a protein such as a myc epitope, GST fusion or His.sub.6 and affinity purified with myc antibody, glutathione resin or Ni-resin, respectively. Such tags can be joined to the nucleotide sequence encoding a soluble PH20 as described elsewhere herein, which can facilitate purification of soluble proteins. For example, a modified PH20 polypeptide can be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification

facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle Wash.). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego, Calif.) between the purification domain and the expressed PH20 polypeptide is useful to facilitate purification. One such expression vector provides for expression of a fusion protein containing a PH20 polypeptide in and an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography), while the enterokinase cleavage site provides a means for purifying the polypeptide from the fusion protein.

~~[0419]~~(314) Purity can be assessed by any method known in the art including gel electrophoresis, orthogonal HPLC methods, staining and ~~spectrophotometry~~spectrophotometric techniques. The expressed and purified protein can be analyzed using any assay or method known to one of skill in the art, for example, any described in Section G. These include assays based on the physical and/or functional properties of the protein, including, but not limited to, analysis by gel electrophoresis, immunoassay and assays of hyaluronidase activity.

~~[0420]~~(315) Depending on the expression system and host cells used, the resulting polypeptide can be heterogeneous due to peptidases present in the culture medium upon production and purification. For example, culture of soluble PH20 in CHO cells can result in a mixture of heterogeneous polypeptides.

~~[0421]~~(316) 6. Modification of Polypeptides by PEGylation

~~[0422]~~(317) Polyethylene glycol (PEG) has been widely used in biomaterials, biotechnology and medicine primarily because PEG is a biocompatible, nontoxic, water-soluble polymer that is typically nonimmunogenic (Zhao and Harris, ACS Symposium Series 680: 458-72, 1997). In the area of drug delivery, PEG derivatives have been widely used in covalent attachment (i.e., ~~"~~“PEGylation~~"~~”) to proteins to reduce immunogenicity, proteolysis and kidney clearance and to enhance solubility (Zalipsky, Adv. Drug Del. Rev. 16:157-82, 1995). Similarly, PEG has been attached to low molecular weight, relatively hydrophobic drugs to enhance solubility, reduce toxicity and alter biodistribution. Typically, PEGylated drugs are injected as solutions.

~~[0423]~~(318) A closely related application is synthesis of crosslinked degradable PEG networks or formulations for use in drug delivery since much of the same chemistry used in design of degradable, soluble drug carriers can also be used in design of degradable gels (Sawhney et al., Macromolecules 26: 581-87, 1993). It also is known that intermacromolecular complexes can be formed by mixing solutions of two complementary polymers. Such complexes are generally stabilized by electrostatic interactions (polyanion-polycation) and/or hydrogen bonds (polyacid-polybase) between the polymers involved, and/or by hydrophobic interactions between the polymers in an aqueous surrounding (Krupers et al., Eur. Polym J. 32:785-790, 1996). For example, mixing solutions of polyacrylic acid (PAAc) and polyethylene oxide (PEO) under the proper conditions results in the formation of complexes based mostly on hydrogen bonding. Dissociation of these complexes at physiologic conditions has been used for delivery of free drugs (i.e., non-PEGylated). In addition, complexes of complementary polymers have been formed from both homopolymers and copolymers.

[0424](319) Numerous reagents for PEGylation have been described in the art. Such reagents include, but are not limited to, reaction of the polypeptide with N-hydroxysuccinimidyl (NHS) activated PEG, succinimidyl mPEG, mPEG2mPEG.sub.2-N-hydroxysuccinimide, mPEG succinimidyl alpha-methylbutanoate, mPEG succinimidyl propionate, mPEG succinimidyl butanoate, mPEG carboxymethyl 3-hydroxybutanoic acid succinimidyl ester, homobifunctional PEG-succinimidyl propionate, homobifunctional PEG propionaldehyde, homobifunctional PEG butyraldehyde, PEG maleimide, PEG hydrazide, p-nitrophenyl-carbonate PEG, mPEG-benzotriazole carbonate, propionaldehyde PEG, mPEG butryaldehyde,butyraldehyde, branched mPEG.sub.2 butyraldehyde, mPEG acetyl, mPEG piperidone, mPEG methylketone, mPEG ""linkerless"" maleimide, mPEG vinyl sulfone, mPEG thiol, mPEG orthopyridylthioester, mPEG orthopyridyl disulfide, Fmoc-PEG-NHS, Boc-PEG-NHS, vinylsulfone PEG-NHS, acrylate PEG-NHS, fluorescein PEG-NHS, and biotin PEG-NHS (see e.g., Monfardini et al., Bioconjugate Chem. 6:62-69, 1995; Veronese et al., J. Bioactive Compatible Polymers 12:197-207, 1997; U.S. Pat. NoNos. 5,672,662; U.S. Pat. No. 5,932,462; U.S. Pat. No. 6,495,659; U.S. Pat. No. 6,737,505; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,179,337; U.S. Pat. No. 5,122,614; U.S. Pat. No. 5,324,844; U.S. Pat. No. 5,446,090; U.S. Pat. No. 5,612,460; U.S. Pat. No. 5,643,575; U.S. Pat. No. 5,766,581; U.S. Pat. No. 5,795,569; U.S. Pat. No. 5,808,096; U.S. Pat. No. 5,900,461; U.S. Pat. No. 5,919,455; U.S. Pat. No. 5,985,263; U.S. Pat. No. 5,990,237; U.S. Pat. No. 6,113,906; U.S. Pat. No. 6,214,966; U.S. Pat. No. 6,258,351; U.S. Pat. No. 6,340,742; U.S. Pat. No. 6,413,507; U.S. Pat. No. 6,420,339; U.S. Pat. No. 6,437,025; U.S. Pat. No. 6,448,369; U.S. Pat. No. 6,461,802; U.S. Pat. No. 6,828,401; U.S. Pat. No. 6,858,736; U.S. 2001/0021763; U.S. 2001/0044526; U.S. 2001/0046481; U.S. 2002/0052430; U.S. 2002/0072573; U.S. 2002/0156047; U.S. 2003/0114647; U.S. 2003/0143596; U.S. 2003/0158333; U.S. 2003/0220447; U.S. 2004/0013637; US 2004/0235734; WO05000360; U.S. 2005/0114037; U.S. 2005/0171328; U.S. 2005/0209416; EP 1064951; EP 0822199; WO 01076640; WO 0002017; WO 0249673; WO 9428024; and WO 0187925).

[0425](320) In one example, the polyethylene glycol has a molecular weight ranging from about 3 kD to about 50 kD, and typically from about 5 kD to about 30 kD. Covalent attachment of the PEG to the drug (known as ""PEGylation"") can be accomplished by known chemical synthesis techniques. For example, the PEGylation of protein can be accomplished by reacting NHS-activated PEG with the protein under suitable reaction conditions.

[0426](321) While numerous reactions have been described for PEGylation, those that are most generally applicable confer directionality, utilize mild reaction conditions, and do not necessitate extensive downstream processing to remove toxic catalysts or bi-products. For instance, monomethoxy PEG (mPEG) has only one reactive terminal hydroxyl, and thus its use limits some of the heterogeneity of the resulting PEG-protein product mixture. Activation of the hydroxyl group at the end of the polymer opposite to the terminal methoxy group is generally necessary to accomplish efficient protein PEGylation, with the aim being to make the derivatised PEG more susceptible to nucleophilic attack. The attacking nucleophile is usually the epsilon-amino group of a lysyl residue, but other amines also can react (e.g., the N-terminal alpha-amine or the ring amines of histidine) if local conditions are favorable. A more directed attachment is possible in proteins containing a single lysine or cysteine. The latter residue can be targeted by PEG-maleimide for thiol-specific modification. Alternatively, PEG hydrazide can be reacted with a periodate oxidized hyaluronan-degrading enzyme and reduced in the presence of NaCNBH.sub.3. More specifically, PEGylated CMP sugars can be reacted with a hyaluronan-

degrading enzyme in the presence of appropriate glycosyl-transferases. One technique is the "“PEGylation"” technique where a number of polymeric molecules are coupled to the polypeptide in question. When using this technique, the immune system has difficulties in recognizing the epitopes on the polypeptide's surface responsible for the formation of antibodies, thereby reducing the immune response. For polypeptides introduced directly into the circulatory system of the human body to give a particular physiological effect (i.e., pharmaceuticals) the typical potential immune response is an IgG and/or IgM response, while polypeptides which are inhaled through the respiratory system (i.e., industrial polypeptide) potentially can cause an IgE response (i.e., allergic response). One of the theories explaining the reduced immune response is that the polymeric molecule(s) shield(s) epitope(s) on the surface of the polypeptide responsible for the immune response leading to antibody formation. Another theory or at least a partial factor is that the heavier the conjugate is, the more reduced the resulting immune response is.

[0427](322) Typically, to make the PEGylated PH20 polypeptide provided herein, PEG moieties are conjugated, via covalent attachment, to the polypeptides. Techniques for PEGylation include, but are not limited to, specialized linkers and coupling chemistries (see e.g., Roberts, Adv. Drug Deliv. Rev. 54:459-476, 2002), attachment of multiple PEG moieties to a single conjugation site (such as via use of branched PEGs; see e.g., Guiotto et al., Bioorg. Med. Chem. Lett. 12:177-180, 2002), site-specific PEGylation and/or mono-PEGylation (see e.g., Chapman et al., Nature Biotech. 17:780-783, 1999), and site-directed enzymatic PEGylation (see e.g., Sato, Adv. Drug Deliv. Rev., 54:487-504, 2002). Methods and techniques described in the art can produce proteins having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 PEG or PEG derivatives attached to a single protein molecule (see e.g., U.S. 2006/0104968).

[0428](323) As an exemplary illustrative method for making a PEGylated PH20 polypeptide, PEG aldehydes, succinimides and carbonates have each been applied to conjugate PEG moieties, typically succinimidyl PEGs, to rHuPH20. For example, rHuPH20 has been conjugated with exemplary succinimidyl monoPEG (mPEG) reagents including mPEG-Succinimidyl Propionates (mPEG-SPA), mPEG-Succinimidyl Butanoates (mPEG-SBA), and (for attaching "“branched"” PEGs) mPEG2-N-Hydroxylsuccinimide. These PEGylated succinimidyl esters contain different length carbon backbones between the PEG group and the activated cross-linker, and either a single or branched PEG group. These differences can be used, for example, to provide for different reaction kinetics and to potentially restrict sites available for PEG attachment to rHuPH20 during the conjugation process.

[0429](324) Succinimidyl PEGs (as above) containing either linear or branched PEGs can be conjugated to PH20. PEGs can used to generate PH20s reproducibly containing molecules having, on the average, between about three to six or three to six PEG molecules per hyaluronidase. Such PEGylated rHuPH20 compositions can be readily purified to yield compositions having specific activities of approximately 25,000 or 30,000 Unit/mg protein hyaluronidase activity, and being substantially free of non-PEGylated PH20 (less than 5% non-PEGylated).

[0430](325) Using various PEG reagents, exemplary versions of a PEGylated PH20 polypeptide can be prepared, for example, using mPEG-SBA (30 kD), mPEG-SMB (30 kD), and branched versions based on mPEG2-NHS (40 kD) and mPEG2-NHS (60 kD). PEGylated versions of PH20 can be generated using NHS chemistries, as well as carbonates, and aldehydes, using each

of the following reagents: mPEG2-NHS-40K branched, mPEG-NHS-10K branched, mPEG-NHS-20K branched, mPEG2-NHS-60K branched; mPEG-SBA-5K, mPEG-SBA-20K, mPEG-SBA-30K; mPEG-SMB-20K, mPEG-SMB-30K; ~~mPEG-butyrldehyde~~mPEG-butyraldehyde; mPEG-SPA-20K, mPEG-SPA-30K; and PEG-NHS-5K-biotin. PEGylated PH20 also can be prepared using PEG reagents available from Dowpharma, a division of Dow Chemical Corporation; including PH20 polypeptides PEGylated with Dowpharma's p-nitrophenyl-carbonate PEG (30 kDa) and with propionaldehyde PEG (30 kDa).

~~[0431]~~(326) In one example, the PEGylation includes conjugation of mPEG-SBA, for example, mPEG-SBA-30K (having a molecular weight of about 30 kDa) or another succinimidyl ester of a PEG butanoic acid derivative, to a PH20 polypeptide. Succinimidyl esters of PEG butanoic acid derivatives, such as mPEG-SBA-30K readily couple to amino groups of proteins. For example, covalent conjugation of m-PEG-SBA-30K and rHuPH20 (which is approximately 60 KDa in size) provides stable amide bonds between rHuPH20 and mPEG, as shown in Scheme 1, below.

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~~[0432]~~(328) Typically, the mPEG-SBA-30K or other PEG is added to the PH20 polypeptide at a PEG:polypeptide molar ratio of 10:1 in a suitable buffer, e.g., 130 mM NaCl/10 mM HEPES at pH 6.8 or 70 mM phosphate buffer, pH 7, followed by sterilization, e.g., sterile filtration, and continued conjugation, for example, with stirring, overnight at 4~~.degree.~~° C. in a cold room. In one example, the conjugated PEG-PH20 is concentrated and buffer-exchanged.

~~[0433]~~(329) Other methods of coupling succinimidyl esters of PEG butanoic acid derivatives, such as mPEG-SBA-30K are known in the art (see e.g., U.S. Pat. ~~No~~Nos. 5,672,662; ~~U.S. Pat. No.~~ 6,737,505; and U.S. 2004/0235734). For example, a polypeptide, such as a PH20 polypeptide, can be coupled to an NHS activated PEG derivative by reaction in a borate buffer (0.1 M, pH 8.0) for one hour at 4~~.degree.~~° C. The resulting PEGylated protein can be purified by ultrafiltration. Another method reacts polypeptide with mPEG-SBA in deionized water to which triethylamine is added to raise the pH to 7.2-9. The resulting mixture is stirred at room temperature for several hours to complete the PEGylation.

~~[0434]~~(330) Methods for PEGylation of PH20 polypeptides, including, for example, animal-derived hyaluronidases and bacterial hyaluronan-degrading enzymes, are known to one of skill in the art. See, for example, European Patent No. EP 0400472, which describes the PEGylation of bovine testes hyaluorindase and chondroitin ABC lyase. Also, U.S. Publication No. 2006014968 describes PEGylation of a human hyaluronidase derived from human PH20. For example, the PEGylated hyaluronan-degrading enzyme generally contains at least 3 PEG moieties per molecule. In some examples, the PH20 polypeptide contains three to six PEG molecules. In other examples, the enzyme can have a PEG to protein molar ratio between 5:1 and 9:1, for example, 7:1.

F. ~~Pharmaceutical Compositions and Formulations, Dosages and Administration~~PHARMACEUTICAL COMPOSITIONS AND FORMULATIONS, DOSAGES AND ADMINISTRATION

[0435](331) Pharmaceutical compositions of any of the modified PH20 polypeptides are provided herein for administration. Pharmaceutically acceptable compositions are prepared in view of approvals for a regulatory agency or other agency prepared in accordance with generally recognized pharmacopeia for use in animals and in humans. Typically, the compounds are formulated into pharmaceutical compositions using techniques and procedures well known in the art (see e.g., Ansel Introduction to Pharmaceutical Dosage Forms, Fourth Edition, 1985, 126).

[0436](332) In particular, provided herein are pharmaceutical compositions that are stable as a liquid formulation for prolonged periods of time for at least 1 month at temperatures from or from about 2.degree.° C. to 8.degree.° C., inclusive or for at least 3 days at a temperature from or from about 30.degree.° C. to 42.degree.° C., inclusive. Pharmaceutical compositions, in particular liquid formulations, can be limited by the stability of the active agent, which can be susceptible to effects of storage conditions (time or length of storage, temperature and/or agitation) and/or, formulation components contained in the composition. Hence, the stable pharmaceutical compositions generally contain a modified PH20 polypeptide as described in Section C.1.b that exhibits increased stability manifested as an increased resistance to one or more protein denaturation conditions. Such protein denaturation conditions can include, but are not limited to, elevated temperature greater than or equal to or about 30.degree.° C., agitation, low or no salt, and presence of excipients. The increased stability is characterized by improved storage time, decreased fragmentation, and/or decreased aggregate formation, while still retaining the activity of the active agent(s), e.g., the PH20 hyaluronidase. Such formulations can be provided as "“ready-to use"” liquid formulations without further reconstitution and/or without any requirement for further dilution. In some examples, the formulations also can be prepared in a lyophilized or concentrated form.

[0437](333) Pharmaceutical compositions containing a modified PH20 polypeptide can be co-administered with another therapeutic agent. In such examples, the modified PH20 polypeptides can be formulated separately as a pharmaceutical composition and administered prior to, simultaneously with, intermittently with, or subsequent to a second composition containing an active therapeutic agent. In other examples, modified PH20 polypeptides can be co-formulated with pharmaceutical formulations of other therapeutic agents.

[0438](334) In particular, provided herein are co-formulations containing a modified PH20 polypeptide as described herein and a therapeutic agent that is a chemotherapeutic agent, an analgesic agent, an anti-inflammatory agent, an antimicrobial agent, an amoebicidal agent, a trichomonacidal agent, an anti-parkinson agent, an anti-malarial agent, an anticonvulsant agent, an anti-depressant agent, and antiarthritics agent, an anti-fungal agent, an antihypertensive agent, an antipyretic agent, an anti-parasite agent, an antihistamine agent, an alpha-adrenergic agonist agent, an alpha blocker agent, an anesthetic agent, a bronchial dilator agent, a biocide agent, a bactericide agent, a bacteriostat agent, a beta adrenergic blocker agent, a calcium channel blocker agent, a cardiovascular drug agent, a contraceptive agent, a decongestant agent, a diuretic agent, a depressant agent, a diagnostic agent, a electrolyte agent, a hypnotic agent, a hormone agent, a hyperglycemic agent, a muscle relaxant agent, a muscle contractant agent, an ophthalmic agent, a parasympathomimetic agent, a psychic energizer agent, a sedative agent, a sympathomimetic agent, a tranquilizer agent, an urinary agent, a vaginal agent, a viricide agent, a vitamin agent, a non-steroidal anti-inflammatory agent, an angiotensin converting enzyme inhibitor agent, a polypeptide, a protein, a nucleic acid, a drug, an organic molecule or a sleep inducer. For

example, modified PH20 polypeptides provided herein can be co-formulated with an antibody such as a monoclonal antibody, an Immune Globulin, an antibiotic, a bisphosphonate, a cytokine, a chemotherapeutic agent, a coagulation factor or an insulin. Exemplary therapeutic agents that can be co-formulated with a modified PH20 polypeptide are described in described in Section H. In particular, provided herein are co-formulations containing a modified PH20 polypeptide and an insulin, such as a fast-acting insulin, for example, a regular insulin or a fast-acting (rapid-acting) insulin analog. The co-formulations provided herein include stable co-formulations, whereby the active agents, i.e., the modified PH20 polypeptide and the therapeutic agent, exhibit increased stability and retain activity for prolonged periods as described herein.

[0439](335) Formulations containing PH20 provided herein, including separate formulations thereof and co-formulations, are stable for prolonged periods of time, including at varied temperatures and under varied storage or use conditions such as agitation. For example, the formulations provided herein are stable and retain activity of active agent(s) (e.g., PH20 hyaluronidase) at ""“refrigerator"”” conditions, for example, at 2.degree.° C. to 8.degree.° C., such as at or about 4.degree.° C., for at least at least 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23 months, 24 months, 25 months, 26 months, 27 months, 28 months, 29 months or 30 months or more. In another example, the formulations provided herein are stable and retain activity of active agent(s) (e.g., PH20 hyaluronidase) at room temperature for example at 18.degree.° C. to 32.degree.° C., generally 20.degree.° C. to 32.degree.° C., such as 28.degree.° C. to 32.degree.° C., for at least 2 weeks to 1 year, for example, at least 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, at least 7 months, at least 8 months, at least 9 months, or at least 1 year or more. In a further example, the formulations provided herein are stable and retain activity of active agent(s) (e.g., PH20 hyaluronidase) at elevated temperatures of about or greater than 30.degree.° C., generally from or from about 30.degree.° C. to 42.degree.° C., such as 32.degree.° C. to 37.degree.° C. or 35.degree.° C. to 37.degree.° C. or about or 37.degree.° C. for at least 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 35 days, 40 days, 45 days, 50 days, 60 days or more.

[0440](336) Compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, and sustained release formulations. A composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and other such agents. Topical formulations also are contemplated. The formulation should suit the mode of administration.

[0441](337) 1. Formulations--Liquids, Injectables, and Emulsions

[0442](338) The formulation generally is made to suit the route of administration. Parenteral administration, generally characterized by injection or infusion, either subcutaneously, intramuscularly, intravenously or intradermally is contemplated herein. Preparations for parenteral administration include sterile solutions ready for injection, sterile dry soluble products, such as lyophilized powders, ready to be combined with a solvent just prior to use,

including hypodermic tablets, sterile suspensions ready for injection, sterile dry insoluble products ready to be combined with a vehicle just prior to use and sterile emulsions. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. For example, the compositions containing a modified PH20 polypeptide, formulated separately or co-formulated with another therapeutic agent, can be provided as a pharmaceutical preparation in liquid form as a solution, syrup or suspension. In liquid form, the pharmaceutical preparations can be provided as a concentrated preparation to be diluted to a therapeutically effective concentration before use. Generally, the preparations are provided in a dosage form that does not require dilution for use. In another example, pharmaceutical preparations can be presented in lyophilized form for reconstitution with water or other suitable vehicle before use.

~~[0443]~~(339) Injectables are designed for local and systemic administration. For purposes herein, local administration is desired for direct administration to the affected interstitium. The solutions can be either aqueous or nonaqueous. If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening and solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof.

~~[0444]~~(340) The concentration of the pharmaceutically active compound is adjusted so that an injection or infusion provides an effective amount to produce the desired pharmacological effect. The exact dose depends on the age, weight and condition of the patient or animal as is known in the art. The unit-dose parenteral preparations can be packaged in, for example, an ampoule, a cartridge, a vial or a syringe with a needle. The volume of liquid solution or reconstituted powder preparation, containing the pharmaceutically active compound, is a function of the disease to be treated and the particular article of manufacture chosen for package. All preparations for parenteral administration must be sterile, as is known and practiced in the art. The percentage of active compound contained in such parenteral compositions is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject.

~~[0445]~~(341) Pharmaceutical compositions can include carriers or other excipients. For example, pharmaceutical compositions provided herein can contain any one or more of a diluents(s), adjuvant(s), antiadherent(s), binder(s), coating(s), ~~fillers~~filler(s), ~~flavors~~flavor(s), color(s), lubricant(s), glidant(s), preservative(s), detergent(s), sorbent(s) or ~~sweeteners~~sweetener(s) and a combination thereof or vehicle with which a modified PH20 polypeptide is administered. For example, pharmaceutically acceptable carriers or excipients used in parenteral preparations include aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, local anesthetics, suspending and dispersing agents, emulsifying agents, sequestering or chelating agents and other pharmaceutically acceptable substances. Formulations, including liquid preparations, can be prepared by conventional means with pharmaceutically acceptable additives or excipients.

~~[0446]~~(342) Examples of suitable pharmaceutical carriers are described in ~~"~~“Remington's Pharmaceutical Sciences~~"~~” by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, generally in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. Such pharmaceutical carriers can be sterile liquids, such as water or oils, including those of petroleum, animal,

vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and sesame oil. Water is a typical carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions also can be employed as liquid carriers, particularly for injectable solutions. Examples of aqueous vehicles include Sodium Chloride Injection, Ringers Injection, Isotonic Dextrose Injection, Sterile Water Injection, Dextrose and Lactated Ringers Injection. Nonaqueous parenteral vehicles include fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil and peanut oil. Suspending and dispersing agents include, but are not limited to, sorbitol syrup, cellulose derivatives or hydrogenated edible fats, sodium carboxymethylcellulose, hydroxypropyl methylcellulose and polyvinylpyrrolidone. Emulsifying agents include, but are not limited to, lecithin or acacia. Detergents include, but are not limited to, Polysorbate 80 (TWEEN 80). Non-aqueous vehicles include, but are not limited to, almond oil, oily esters, or fractionated vegetable oils. Anti-microbial agents or preservatives include, but are not limited to, methyl or propyl-p-hydroxybenzoates or sorbic acid, m-cresol, phenol. A diluent includes, but is not limited to, lactose, sucrose, dicalcium phosphate, or carboxymethylcellulose. A lubricant includes, but is not limited to, magnesium stearate, calcium stearate or talc. A binder includes, but is not limited to, starch, natural gums, such as gum acacia, gelatin, glucose, molasses, polyvinylpyrrolidine, celluloses and derivatives thereof, povidone, crospovidones and other such binders known to those of skill in the art. Isotonic agents include, but are not limited to, sodium chloride and dextrose. Buffers include, but are not limited to, phosphate and citrate. Antioxidants include sodium bisulfate. Local anesthetics include procaine hydrochloride. A sequestering or chelating agent of metal ions includes EDTA. Other suitable pharmaceutical excipients include, but are not limited to, starch, glucose, lactose, dextrose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, saline, water, and ethanol. Pharmaceutical carriers also include ethyl alcohol, polyethylene glycol and propylene glycol for water miscible vehicles and sodium hydroxide, hydrochloric acid, citric acid or lactic acid for pH adjustment. A composition, if desired, also can contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, or pH buffering agents, for example, acetate, sodium citrate, cyclodextrin derivatives, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, stabilizers, solubility enhancers, and other such agents such as for example, sodium acetate, sodium phosphate, sorbitan monolaurate, triethanolamine oleate and cyclodextrins.

~~[0447]~~(343) In particular, antimicrobial agents (e.g., preservatives) in bacteriostatic or fungistatic concentrations (e.g., an anti-microbial effective amount) can be added to parenteral preparations packaged in multiple-dose containers, which include phenols or cresols, mercurials, benzyl alcohol, chlorobutanol, methyl and propyl p-hydroxybenzoic acid esters, thimerosal, benzalkonium chloride and benzethonium chloride.

~~[0448]~~(344) The volume of the formulations, including the separately formulated or co-formulated PH20-containing formulations provided herein, can be any volume suitable for the container in which it is provided. In some examples, the formulations are provided in a vial, syringe, pen, reservoir for a pump or a closed loop system, or any other suitable container. For example, the formulations provided herein are between or about between 0.1 mL to 500 mL, such as 0.1 mL to 100 mL, 1 mL to 100 mL, 0.1 mL to 50 mL, such as at least or about at least or about or 0.1 mL, 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 10 mL, 15 mL, 20 mL, 30 mL, 40 mL, 50 mL or more.

[0449](345) a. Lyophilized Powders

[0450](346) Of interest herein are lyophilized powders, which can be reconstituted for administration as solutions, emulsions and other mixtures. They may also be reconstituted and formulated as solids or gels.

[0451](347) The sterile, lyophilized powder is prepared by dissolving a compound of enzyme in a buffer solution. The buffer solution may contain an excipient which improves the stability or other pharmacological component of the powder or reconstituted solution, prepared from the powder. Subsequent sterile filtration of the solution followed by lyophilization under standard conditions known to those of skill in the art provides the desired formulation. A liquid formulation as described herein above can be prepared. The resulting mixture is sterile filtered or treated to remove particulates and to insure sterility, and apportioned into vials for lyophilization. For example, the lyophilized powder can be prepared by dissolving an excipient, such as dextrose, sorbitol, fructose, corn syrup, xylitol, glycerin, glucose, sucrose or other suitable agent, in a suitable buffer, such as citrate, sodium or potassium phosphate or other such buffer known to those of skill in the art. Then, a selected enzyme is added to the resulting mixture, and stirred until it dissolves.

[0452](348) Each vial is made to contain a single dosage or multiple dosages of the compound. The lyophilized powder can be stored under appropriate conditions, such as at about 4.degree.° C. to room temperature. Reconstitution of this lyophilized powder with an appropriate buffer solution provides a formulation for use in parenteral administration.,.

[0453](349) b. Exemplary Formulations

[0454](350) Single dose formulations of PH20 are known in the art. For example, Hylenex.RTM.® recombinant (hyaluronidase human injection) contains, per mL, 8.5 mg NaCl (145 mM), 1.4 mg dibasic sodium phosphate (9.9 mM), 1.0 mg human albumin, 0.9 mg edetate disodium (2.4 mM), 0.3 mg CaCl.sub.2 (2.7 mM) and NaOH to adjust the pH to 7.4. Other formulations of human soluble hyaluronidase, such as the rHuPH20 formulations described in U.S. Pat. Pub. No. US2011/0053247, include 130 mM NaCl, 10 mM Hepes, pH 7.0; or 10 mM histidine, 130 mM NaCl, pH 6.0. Any of the modified PH20 polypeptides provided herein can be similarly formulated.

[0455](351) In addition to a therapeutically effective amount of a modified PH20 polypeptide and/or other therapeutic agent, exemplary pharmaceutical compositions provided herein, including separately formulated- and co-formulated-PH20 containing formulations, can contain a concentration of NaCl and are prepared at a requisite pH to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase and/or other co-formulated therapeutic agent). For multi-dose formulations and other formulations stored for a prolonged time, the compositions generally also contain one or more preservatives. Further stabilizing agents and other excipients also can be included. Exemplary components are described below.

[0456](352) i. Salt (e.g. NaCl)

[0457](353) In examples herein, the pharmaceutical compositions provided herein contain a concentration of salt, such as sodium chloride (NaCl), to maintain the stability of the active

agent(s) (e.g., PH20 hyaluronidase). Salt, such as NaCl, is generally required to retain PH20 stability and activity. Low salt concentrations of generally less than 120 mM can have deleterious effects on PH20 activity over time and depending on temperature conditions. Hence, the absence of salt (e.g. NaCl) or a low concentration of salt (e.g. NaCl) can result in instability of the protein. In some examples herein, however, modified PH20 polypeptides that exhibit increased stability in the absence of low or no salt, such as low or no NaCl (see e.g., Section C.1.b.iii), are not susceptible to denaturation. Also, the presence of salt (e.g. NaCl) can have differing effects on other therapeutic agents. For example, the solubility of insulin and insulin analogs tends to increase with lower salt concentration (e.g., ~~<~~≤140 mM) and high salt concentrations can result in crystallization/aggregation of insulin, especially at lower temperatures (see e.g., U.S. Provisional Appl. No. 61/520,962; U.S. application Ser. Nos. 13/507,263 and 13/507,262; and International PCT Application No. PCT/US2012/042816). Thus, pharmaceutical compositions provided herein are prepared in accordance with the requirements of the active agent(s). It is within the level of one of skill in the art to assess the stability of the active agent(s) in the formulation and under various storage conditions (see e.g., Section G). In particular examples herein, the pharmaceutical compositions, including the separately formulated or co-formulated PH20-containing formulations provided herein, contain NaCl at a concentration of between or about between 10 mM to 200 mM, such as 10 mM to 50 mM, 50 mM to 200 mM, 50 mM to 120 mM, 50 mM to 100 mM, 50 mM to 90 mM, 120 mM to 160 mM, 130 mM to 150 mM, 80 mM to 140 mM, 80 mM to 120 mM, 80 mM to 100 mM, 80 mM to 160 mM, 100 mM to 140 mM, 120 mM to 120 mM or 140 mM to 180 mM.

~~[0458]~~(354) ii. pH and Buffer

~~[0459]~~(355) In examples herein, the pharmaceutical compositions provided herein are prepared at a pH to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase). For example, the pharmaceutical compositions provided herein are prepared at a pH of between or about between 6.5 to 7.8 such as between or about between 6.5 to 7.2, 7.0 to 7.8, 7.0 to 7.6 or 7.2 to 7.4. Reference to pH herein is based on measurement of pH at room temperature. It is understood that the pH can change during storage over time, but typically will remain between or between about pH 6.5 to or to about 7.8. For example, the pH can vary by ~~.+-.~~±0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.3, 1.4, 1.5 or more. Exemplary co-formulations provided herein have a pH of or of about 7.0~~.+-.~~±0.2, 7.1~~.+-.~~±0.2, 7.2~~.+-.~~±0.2, 7.3~~.+-.~~±0.2, 7.4~~.+-.~~±0.2, 7.5~~.+-.~~±0.2 or 7.6~~.+-.~~±0.2 when prepared. If necessary, pH can be adjusted using acidifying agents to lower the pH or alkalizing agents to increase the pH. Exemplary acidifying agents include, but are not limited to, acetic acid, citric acid, sulfuric acid, hydrochloric acid, monobasic sodium phosphate solution, and phosphoric acid. Exemplary alkalizing agents include, but are not limited to, dibasic sodium phosphate solution, sodium carbonate, or sodium hydroxide.

~~[0460]~~(356) The compositions are generally prepared using a buffering agent that maintains the pH range. Any buffer can be used in formulations provided herein so long as it does not adversely affect the stability of the active agent(s) (e.g., PH20 hyaluronidase), and supports the requisite pH range required. Examples of particularly suitable buffers include Tris, succinate, acetate, phosphate buffers, citrate, aconitate, malate and carbonate. Those of skill in the art, however, will recognize that formulations provided herein are not limited to a particular buffer, so long as the buffer provides an acceptable degree of pH stability, or "“buffer capacity"” in the range indicated. Generally, a buffer has an adequate buffer capacity within about 1 pH unit of its

pK (Lachman et al. In: The Theory and Practice of Industrial Pharmacy 3rd Edn. (Lachman, L., Lieberman, H A. and Kanig, J. L., Eds.), Lea and Febiger, Philadelphia, p. 458-460, 1986). Buffer suitability can be estimated based on published pK tabulations or can be determined empirically by methods well known in the art. The pH of the solution can be adjusted to the desired endpoint within the range as described above, for example, using any acceptable acid or base.

~~[0461]~~(357) Buffers that can be included in the co-formulations provided herein include, but are not limited to, Tris (Tromethamine), histidine, phosphate buffers, such as dibasic sodium phosphate, and citrate buffers. Such buffering agents can be present in the co-formulations at concentrations between or about between 1 mM to 100 mM, such as 10 mM to 50 mM or 20 mM to 40 mM, such as at or about 30 mM. For example, such buffering agents can be present in the co-formulations in a concentration of or about 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM, 60 mM, 65 mM, 70 mM, 75 mM, or more.

~~[0462]~~(358) iii. Preservative(s)

~~[0463]~~(359) In examples herein, multi-dose formulations or formulations stored for prolonged periods contain an anti-microbially effective amount of preservative or mixture of preservatives in an amount to have a bacteriostatic or fungistatic effect. In particular examples, the preservatives are present in a sufficient concentration to provide the anti-microbial requirements of, for example, the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP), including the EP anti-microbial requirements (EPA) and the preferred EP anti-microbial requirements (EPB) (see Table 4). Since the presence of preservatives, and in particular phenolic preservatives, can have deleterious effects on the stability of PH20, such formulations typically contain a modified PH20 polypeptide that exhibits increased stability in the presence of preservatives, such as any described in Section C.1.b.i herein. Generally, the amount maintains the stability of the active agent(s) (e.g., PH20 hyaluronidase).

~~[0464]~~(360) An anti-microbial effective amount of preservative is an amount that exhibits anti-microbial activity by killing or inhibiting the propagation of microbial organisms in a sample of the composition as assessed in an antimicrobial preservative effectiveness test (APET). One of skill in the art is familiar with the antimicrobial preservative effectiveness test and standards to be meet under the USP and EPA or EPB in order to meet minimum requirements. In general, the antimicrobial preservative effectiveness test involves challenging a composition with prescribed inoculums of suitable microorganisms, i.e., bacteria, yeast and fungi, storing the inoculated preparation at a prescribed temperature, withdrawing samples at specified intervals of time and counting the organisms in the sample (see, Sutton and Porter, (2002) PDA Journal of Pharmaceutical Science and Technology 56(4):300-311; The United States Pharmacopeial Convention, Inc., (effective Jan. 1, 2002), The United States Pharmacopeia 25.sup.th Revision, Rockville, Md., Chapter ~~<~~<51~~>~~> Antimicrobial Effectiveness Testing; and European Pharmacopoeia, Chapter 5.1.3, Efficacy of Antimicrobial Preservation). The microorganisms used in the challenge generally include three strains of bacteria, namely E. coli (ATCC No. 8739), Pseudomonas aeruginosa (ATCC No. 9027) and Staphylococcus aureus (ATCC No. 6538), yeast (Candida albicans ATCC No. 10231) and fungus (Aspergillus niger ATCC No.

16404), all of which are added such that the inoculated composition contains 10.sup.5 or 10.sup.6 colony forming units (cfu) of microorganism per mL of composition. The preservative properties of the composition are deemed adequate if, under the conditions of the test, there is a significant fall or no increase, as specified in Table 3 in the number of microorganisms in the inoculated composition after the times and at the temperatures prescribed. The criteria for evaluation are given in terms of the log reduction in the number of viable microorganism as compared to the initial sample or the previous time point.

[0465](361) Non-limiting examples of preservatives that can be included in the co-formulations provided herein include, but are not limited to, phenol, meta-cresol (m-cresol), methylparaben, benzyl alcohol, thimerosal, benzalkonium chloride, 4-chloro-1-butanol, chlorhexidine dihydrochloride, chlorhexidine digluconate, L-phenylalanine, EDTA, bronopol (2-bromo-2-nitropropane-1,3-diol), phenylmercuric acetate, glycerol (glycerin), imidurea, chlorhexidine, sodium dehydroacetate, ortho-cresol (o-cresol), para-cresol (p-cresol), chlorocresol, cetrimide, benzethonium chloride, ethylparaben, propylparaben or butylparaben and any combination thereof. For example, formulations provided herein can contain a single preservative. In other examples, the formulations contain at least two different preservatives or at least three different preservatives. For example, formulations provided herein can contain two preservatives such as L-phenylalanine and m-cresol, L-phenylalanine and methylparaben, L-phenylalanine and phenol, m-cresol and methylparaben, phenol and methylparaben, m-cresol and phenol or other similar combinations. In one example, the preservative in the formulation contains at least one phenolic preservative. For example, the formulation contains phenol, m-cresol or phenol and m-cresol.

[0466](362) In the formulations provided herein, the total amount of the one or more preservative agents as a percentage (%) of mass concentration (w/v) in the formulation can be, for example, between from or between about from 0.1% to 0.4%, such as 0.1% to 0.3%, 0.15% to 0.325%, 0.15% to 0.25%, 0.1% to 0.2%, 0.2% to 0.3%, or 0.3% to 0.4%. Generally, the formulations contain less than 0.4% (w/v) preservative. For example, the co-formulations provided herein contain at least or about at least 0.1%, 0.12%, 0.125%, 0.13%, 0.14%, 0.15%, 0.16% 0.17%, 0.175%, 0.18%, 0.19%, 0.2%, 0.25%, 0.3%, 0.325%, 0.35% but less than 0.4% total preservative.

[0467](363) In some examples, the formulations provided herein contain between or between about 0.1% to 0.25% phenol and between or about between 0.05% to 0.2% m-cresol, such as between or about between 0.10% to 0.2% phenol and between or about between 0.06% to 0.18% m-cresol, or between or about between 0.1% to 0.15% phenol and between or about between 0.08% to 0.15% m-cresol. For example, formulations provided herein contain or contain about 0.1% phenol and 0.075% m-cresol; 0.1% phenol and 0.15% m-cresol; 0.125% phenol and 0.075% m-cresol; 0.13% phenol and 0.075% m-cresol; 0.13% phenol and 0.08% m-cresol; 0.15% phenol and 0.175% m-cresol; or 0.17% phenol and 0.13% m-cresol.

[0468](364) iv. Stabilizers

[0469](365) In examples herein, the pharmaceutical compositions provided herein optionally can contain one or more other stabilizing agent to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase). Included among the types of stabilizers that can be contained in the formulations provided herein are amino acids, amino acid derivatives, amines, sugars, polyols,

salts and buffers, surfactants, and other agents. The formulations provided herein contain at least one stabilizer. For example, the formulations provided herein contain at least one, two, three, four, five, six or more stabilizers. Hence, any one or more of an amino acids, amino acid derivatives, amines, sugars, polyols, salts and buffers, surfactants, and other agents can be included in the formulations herein. Generally, the formulations herein contain at least contain a surfactant and an appropriate buffer. Optionally, the formulations provided herein can contain other additional stabilizers. Other components include, for example, one or more tonicity modifiers, one or more anti-oxidation agents, or other stabilizer.

~~[0470]~~(366) Exemplary amino acid stabilizers, amino acid derivatives or amines include, but are not limited to, L-Arginine, Glutamine, Glycine, Lysine, Methionine, Proline, Lys-Lys, Gly-Gly, Trimethylamine oxide (TMAO) or betaine. Exemplary sugars and polyols include, but are not limited to, glycerol, sorbitol, mannitol, inositol, sucrose or trehalose. Exemplary salts and buffers include, but are not limited to, magnesium chloride, sodium sulfate, Tris such as Tris (100 mM), or sodium Benzoate. Exemplary surfactants include, but are not limited to, poloxamer 188 (e.g., Pluronic~~.RTM.~~® F68), polysorbate 80 (PS80), polysorbate 20 (PS20). Other stabilizers include, but are not limited to, hyaluronic acid (HA), human serum albumin (HSA), phenyl butyric acid, taurocholic acid, polyvinylpyrolidone (PVP) or zinc.

~~[0471]~~(367) In particular examples herein, the formulations contain one or more detergents, such as surfactants, to maintain the stability of the active agent(s) (e.g., PH20 hyaluronidase). For example, surfactants can inhibit aggregation of PH20 and minimize absorptive loss. The surfactants generally are non-ionic surfactants. Surfactants that can be included in the formulations herein include, but are not limited to, partial and fatty acid esters and ethers of polyhydric alcohols such as of glycerol, or sorbitol, poloxamers and polysorbates. For example, exemplary surfactants in the ~~-~~formulations herein include any one or more of poloxamer 188 (PLURONICS~~.RTM.~~® such as PLURONIC~~.RTM.~~® F68), TETRONICS~~.RTM.~~®, polysorbate 20, polysorbate 80, PEG 400, PEG 3000, Tween~~.RTM.~~® (e.g., Tween~~.RTM.~~® 20 or Tween~~.RTM.~~® 80), Triton~~.RTM.~~® X-100, SPAN~~.RTM.~~®, MYRJ~~.RTM.~~®, BRIJ~~.RTM.~~®, CREMOPHOR~~.RTM.~~®, polypropylene glycols or polyethylene glycols. In some examples, the formulations herein contain poloxamer 188, polysorbate 20, polysorbate 80, generally poloxamer 188 (pluronic F68). The formulations provided herein generally contain at least one surfactant, such as 1, 2 or 3 surfactants.

~~[0472]~~(368) In the formulations provided herein, the total amount of the one or more surfactants as a percentage (%) of mass concentration (w/v) in the formulation can be, for example, between from or between about from 0.005% to 1.0%, such as between from or between about from 0.01% to 0.5%, such as 0.01% to 0.1% or 0.01% to 0.02%. Generally, the formulations contain at least 0.01% surfactant and contain less than 1.0%, such as less than 0.5% or less than 0.1% surfactant. For example, the formulations provided herein can contain at or about 0.001%, 0.005%, 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.055%, 0.06%, 0.065%, 0.07%, 0.08%, or 0.09% surfactant. In particular examples, the formulations provided herein contain or contain about 0.01% to or to about 0.05% surfactant.

~~[0473]~~(369) Tonicity modifiers can be included in the formulation provided herein to produce a solution with the desired osmolality. The formulations provided herein have an osmolality of between or about between 245 mOsm/kg to 305 mOsm/kg. For example, the osmolality is or is

about 245 mOsm/kg, 250 mOsm/kg, 255 mOsm/kg, 260 mOsm/kg, 265 mOsm/kg, 270 mOsm/kg, 275 mOsm/kg, 280 mOsm/kg, 285 mOsm/kg, 290 mOsm/kg, 295 mOsm/kg, 300 mOsm/kg or 305 mOsm/kg. In some examples, the formulations have an osmolality of or of about 275 mOsm/kg. Tonicity modifiers include, but are not limited to, glycerin, NaCl, amino acids, polyalcohols, trehalose, and other salts and/or sugars. The particular amount can be empirically determined in order to retain enzyme activity, and/or tonicity.

~~[0474]~~(370) In other instances, glycerin (glycerol) is included in the formulations. For example, formulations provided herein typically contain less than 60 mM glycerin, such as less than 55 mM, less than 50 mM, less than 45 mM, less than 40 mM, less than 35 mM, less than 30 mM, less than 25 mM, less than 20 mM, less than 15 mM, 10 mM or less. The amount of glycerin typically depends on the amount of NaCl present: the more NaCl present in the formulation, the less glycerin is required to achieve the desired osmolality or osmolarity. Thus, for example, in formulations containing higher NaCl concentrations, little or no glycerin need be included in the formulation. In contrast, in formulations containing slightly lower NaCl concentrations, glycerin can be included. For example, formulations provided herein can contain glycerin at a concentration of 40 mM to 60 mM, such as less than 50 mM, such as 20 mM to 50 mM, for example at or about 50 mM.

~~[0475]~~(371) The formulations provided herein also can contain antioxidants to reduce or prevent oxidation, in particular oxidation of the PH20 polypeptide. For example, oxidation can be effected by high concentrations of surfactant or hyaluronan oligomers. Exemplary antioxidants include, but are not limited to, cysteine, tryptophan and methionine. In particular examples, the ~~antioxidant~~anti-oxidant is methionine. The formulations provided herein can include an antioxidant at a concentration from between or from about between 5 mM to or to about 50 mM, such as 5 mM to 40 mM, 5 mM to 20 mM or 10 mM to 20 mM. For example, methionine can be provided in the formulations herein at a concentration from between or from about between 5 mM to or to about 50 mM, such as 5 mM to 40 mM, 5 mM to 20 mM or 10 mM to 20 mM. For example, an antioxidant, for example methionine, can be included at a concentration that is or is about 5 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM, 21 mM, 22 mM, 23 mM, 24 mM, 25 mM, 26 mM, 27 mM, 28 mM, 29 mM, 30 mM, 35 mM, 40 mM, 45 mM or 50 mM. In some examples, the formulations contain 10 mM to 20 mM methionine, such as or about 10 mM or 20 mM methionine.

~~[0476]~~(372) The formulations provided herein also can contain an amino acid stabilizer, which contributes to the stability of the preparation. The stabilizer can be a non-polar or basic amino acid. Exemplary non-polar and basic amino acids include, but are not limited to, alanine, histidine, arginine, lysine, ornithine, isoleucine, valine, methionine, glycine and proline. For example, the amino acid stabilizer is glycine or proline, typically glycine. The stabilizer can be a single amino acid or it can be a combination of 2 or more such amino acids. The amino acid stabilizers can be natural amino acids, amino acid analogues, modified amino acids or amino acid equivalents. Generally, the amino acid is an L-amino acid. For example, when proline is used as the stabilizer, it is generally L-proline. It is also possible to use amino acid equivalents, for example, proline analogues. The concentration of amino acid stabilizer, for example glycine, included in the formulation ranges from 0.1 M to 1 M amino acid, typically 0.1 M to 0.75 M, generally 0.2 M to 0.5 M, for example, at least at or about 0.1 M, 0.15 M, 0.2 M, 0.25 M, 0.3 M, 0.35 M, 0.4 M, 0.45 M, 0.5 M, 0.6 M, 0.7 M, 0.75 M or more amino acid. The amino acid, for

example glycine, can be used in a form of a pharmaceutically acceptable salt, such as hydrochloride, hydrobromide, sulfate, acetate, etc. The purity of the amino acid, for example glycine, should be at least 98%, at least 99%, or at least 99.5% or more.

[0477](373) In examples herein, if necessary, hyaluronidase inhibitors are included in a formulation to stabilize PH20, in particular to reduce the effects of otherwise destabilizing agents and conditions, such as, for example, low salt, high pH, the presence of preservatives and elevated temperatures, present in the formulation. Such a component generally is not required for pharmaceutical compositions containing a modified PH20 polypeptide as provided herein that exhibits increased stability under such conditions. When provided, the hyaluronidase inhibitor is provided at least at its equilibrium concentration. One of skill in the art is familiar with various classes of hyaluronidase inhibitors (see e.g., Girish et al. (2009) Current Medicinal Chemistry, 16:2261-2288, and references cited therein). One of skill in the art knows or can determine by standard methods in the art the equilibrium concentration of a hyaluronidase inhibitor in a reaction or stable composition herein.

[0478](374) An exemplary hyaluronidase inhibitor for use in the compositions herein is hyaluronan (HA). Hyaluronic acid (HA, also known as hyaluronan and hyaluronate) is the natural substrate for PH20. HA is a non-sulfated glycosaminoglycan that is widely distributed throughout connective, epithelial, and neural tissues. It is a polymer of up to 25,000 disaccharide units, themselves composed of D-glucuronic acid and D-N-acetylglucosamine. The molecular weight of HA ranges from about 5 kDa to 200,000 kDa. Any size HA can be used in the compositions as a stabilizer. In some examples, the HA is a disaccharide, composed of D-glucuronic acid and D-N-acetylglucosamine. In other examples, the HA is an oligosaccharide, such as a tetrasaccharide, containing 2 repeating disaccharide units, or alternatively, the HA used in the co-formulations provided herein can contain multiple repeating disaccharide units, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30 or more disaccharide units. In another example, the HA used in the formulations provided herein has a molecular weight that is from or from about 5 kDa to or to about 5,000 kDa; from or from about 5 kDa to or to about 1,000 kDa; from or from about 5 kDa to or to about 500 kDa; or from or from about 5 kDa to or to about 200 kDa. Exemplary HA oligosaccharides for use in the formulations herein have a molecular weight of or of about 6.4 kDa, 74.0 kDa, or 234.4 kDa. The formulations can contain 1 mg/mL to 20 mg/mL HA, 8 mg/mL to 12 mg/mL, such as at least or about 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14 mg/mL, 15 mg/mL, 16 mg/mL, 17 mg/mL, 18 mg/mL, 19 mg/mL or 20 mg/mL or more HA. In some examples, the molar ratio of HA to PH20 is or is about 100,000:1, 95,000:1, 90,000:1, 85,000:1, 80,000:1, 75,000:1, 70,000:1, 65,000:1, 60,000:1, 55,000:1, 50,000:1, 45,000:1, 40,000:1, 35,000:1, 30,000:1, 25,000:1, 20,000:1, 15,000:1, 10,000:1, 5,000:1, 1,000:1, 900:1,8001, 800:1, 700:1, 600:1, 500:1, 400:1, 300:1, 200:1, or 100:1 or less.

[0479](375) In some examples, a nicotinic compound is used as a stabilizing agent. Nicotinic compounds include, but are not limited to, nicotinamide, nicotinic acid, niacin, niacinamide, vitamin B3 and/or salts thereof and/or any combination thereof. In particular applications, the stabilizing agent can include a nicotinic compound an amino acid or amino acids (see e.g., International Publication No. WO2010149772). For example, the amino acid can be arginine, glutamic acid and/or salts thereof or combinations thereof.

~~[0480]~~(376) 2. Compositions for Other Routes of Administration

~~[0481]~~(377) Depending upon the condition treated other routes of administration, such as topical application, transdermal patches, oral and rectal administration are also contemplated herein.

~~[0482]~~(378) For example, pharmaceutical dosage forms for rectal administration are rectal suppositories, capsules and tablets for systemic effect. Rectal suppositories include solid bodies for insertion into the rectum which melt or soften at body temperature releasing one or more pharmacologically or therapeutically active ingredients. Pharmaceutically acceptable substances utilized in rectal suppositories are bases or vehicles and agents to raise the melting point. Examples of bases include cocoa butter (theobroma oil), glycerin-gelatin, carbowax (polyoxyethylene glycol) and appropriate mixtures of mono-, di- and triglycerides of fatty acids. Combinations of the various bases may be used. Agents to raise the melting point of suppositories include spermaceti and wax. Rectal suppositories may be prepared either by the compressed method or by molding. The typical weight of a rectal suppository is about 2 to 3 gm. Tablets and capsules for rectal administration are manufactured using the same pharmaceutically acceptable substance and by the same methods as for formulations for oral administration. Formulations suitable for rectal administration can be provided as unit dose suppositories. These can be prepared by admixing the active compound with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

~~[0483]~~(379) For oral administration, pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets can be coated by methods well-known in the art.

~~[0484]~~(380) Formulations suitable for buccal (sublingual) administration include, for example, lozenges containing the active compound in a flavored base, usually sucrose and acacia or tragacanth; and pastilles containing the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

~~[0485]~~(381) Topical mixtures are prepared as described for the local and systemic administration. The resulting mixtures can be solutions, suspensions, emulsion or the like and are formulated as creams, gels, ointments, emulsions, solutions, elixirs, lotions, suspensions, tinctures, pastes, foams, aerosols, irrigations, sprays, suppositories, bandages, dermal patches or any other formulations suitable for topical administration.

~~[0486]~~(382) The compounds or pharmaceutically acceptable derivatives thereof may be formulated as aerosols for topical application, such as by inhalation (see, e.g., U.S. Pat. Nos. 4,044,126, 4,414,209, and 4,364,923, which describe aerosols for delivery of a steroid useful for treatment of inflammatory diseases, particularly asthma). These formulations, for administration to the respiratory tract, can be in the form of an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose.

In such a case, the particles of the formulation will typically have diameters of less than 50 microns, or less than 10 microns.

[0487](383) The compounds can be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Topical administration is contemplated for transdermal delivery and also for administration to the eyes or mucosa, or for inhalation therapies. Nasal solutions of the active compound alone or in combination with other pharmaceutically acceptable excipients also can be administered.

[0488](384) Formulations suitable for transdermal administration are provided. They can be provided in any suitable format, such as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches contain the active compound in an optionally buffered aqueous solution of, for example, 0.1 to 0.2 M concentration with respect to the active compound. Formulations suitable for transdermal administration also can be delivered by iontophoresis (see, e.g., Tyle, P, Pharmaceutical Research 3(6):318-326 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound.

[0489](385) Pharmaceutical compositions also can be administered by controlled release formulations and/or delivery devices (see e.g., in U.S. Pat. Nos. 3,536,809; 3,598,123; 3,630,200; 3,845,770; 3,916,899; 4,008,719; 4,769,027; 5,059,595; 5,073,543; 5,120,548; 5,591,767; 5,639,476; 5,674,533 and 5,733,566).

[0490](386) 3. Dosages and Administration

[0491](387) The modified PH20 polypeptides provided herein can be formulated as pharmaceutical compositions for single dosage or multiple dosage administration. The PH20 polypeptide is included in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. The therapeutically effective concentration can be determined empirically by testing the polypeptides in known in vitro and in vivo systems such as by using the assays provided herein or known in the art (see e.g., Taliani et al. (1996) Anal. Biochem., 240: 60-67; Filocamo et al. (1997) J Virology, 71: 1417-1427; Sudo et al. (1996) Antiviral Res. 32: 9-18; Bouffard et al. (1995) Virology, 209:52-59; Bianchi et al. (1996) Anal. Biochem., 237: 239-244; Hamatake et al. (1996) Intervirology 39:249-258; Steinkuhler et al. (1998) Biochem., 37:8899-8905; D'Souza et al. (1995) J. Gen. Virol., 76:1729-1736; Takeshita et al. (1997) Anal. Biochem., 247:242-246; see also e.g., Shimizu et al. (1994) J. Virol. 68:8406-8408; Mizutani et al. (1996) J. Virol. 70:7219-7223; Mizutani et al. (1996) Biochem. Biophys. Res. Commun., 227:822-826; Lu et al. (1996) Proc. Natl. Acad. Sci (USA), 93:1412-1417; Hahm et al., (1996) Virology, 226:318-326; Ito et al. (1996) J. Gen. Virol., 77:1043-1054; Mizutani et al. (1995) Biochem. Biophys. Res. Commun., 212:906-911; Cho et al. (1997) J. Virol. Meth. 65:201-207 and then extrapolated therefrom for dosages for humans.

[0492](388) The amount of a modified PH20 to be administered for the treatment of a disease or condition can be determined by standard clinical techniques. In addition, in vitro assays and animal models can be employed to help identify optimal dosage ranges. The precise dosage,

which can be determined empirically, can depend on the particular enzyme, the route of administration, the type of disease to be treated and the seriousness of the disease.

~~[0493]~~(389) Hence, it is understood that the precise dosage and duration of treatment is a function of the disease being treated and can be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values also can vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or use of compositions and combinations containing them. The compositions can be administered hourly, daily, weekly, monthly, yearly or once. Generally, dosage regimens are chosen to limit toxicity. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity, or bone marrow, liver or kidney or other tissue dysfunctions. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

~~[0494]~~(390) Typically, a therapeutically effective dose of a modified PH20 enzyme is at or about 10 Unit (U) to 500,000 Units, 100 Units to 100,000 Units, 500 Units to 50,000 Units, 1000 Units to 10,000 Units, 5000 Units to 7500 Units, 5000 Units to 50,000 Units, or 1,000 Units to 10,000 Units, generally 1,000 to 50,000 Units, in a stabilized solution or suspension or a lyophilized form. For example, a PH20 polypeptide, can be administered at a dose of at least or about at least or 10 U, 20 U, 30 U, 40 U, 50 U, 100 U, 150 U, 200 U, 250 U, 300 U, 350 U, 400 U, 450 U, 500 U, 600 U, 700 U, 800 U, 900 U, 1000 U, 2,000 U, 3,000 U, 4,000 Units, 5,000 U or more. The formulations can be provided in unit-dose forms such as, but not limited to, ampoules, syringes and individually packaged tablets or capsules.

~~[0495]~~(391) The PH20 enzyme can be administered alone, or with other pharmacologically effective agent(s) or therapeutic agent(s), in a total volume of 0.1-100 mL, 1-50 mL, 10-50 mL, 10-30 mL, 1-20 mL, or 1-10 mL, typically 10-50 mL. Typically, volumes of injections or infusions of a PH20-containing composition are at least or at least about 0.01 mL, 0.05 mL, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, 10 mL, 20 mL, 30 mL, 40 mL, 50 mL or more. The formulations provided herein contain a modified PH20 polypeptide in an amount between or about between 30 Units/mL to 3000 U/mL, 300 U/mL to 2000 U/mL or 600 U/mL to 2000 U/mL or 600 U/mL to 1000 U/mL, such as at least or about at least 30 U/mL, 35 U/mL, 40 U/mL, 50 U/mL, 100 U/mL, 200 U/mL, 300 U/mL, 400 U/mL, 500 U/mL, 600 U/mL, 700 U/mL, 800 U/mL, 900 U/mL, 1000 U/mL, 2000 U/mL or 3000 U/mL. For example, the formulations provided herein contain a PH20 that is in an amount that is at least 100 U/mL to 1000 U/mL, for example at least or about at least or about or 600 U/mL.

~~[0496]~~(392) The PH20 polypeptide can be provided as a solution in an amount that is at least or about or is 100 U/mL, 150 U/mL, 200 U/mL, 300 U/mL, 400 U/mL, 500 U/mL, 600 U/mL, 800 U/mL or 1000 U/mL, or can be provided in a more concentrated form, for example in an amount that is at least or about or is 2000 U/mL, 3000 Units/mL, 4000 U/mL, 5000 U/mL, 8000 U/mL,

10,000 U/mL or 20,000 U/mL for use directly or for dilution to the effective concentration prior to use. The PH20 polypeptide compositions can be provided as a liquid or lyophilized formulation.

~~[0497]~~(393) When the PH20 is co-formulated with a therapeutic agent, dosages can be provided as a ratio of the amount of a PH20 polypeptide to the amount of therapeutic agent administered. For example, a PH20 polypeptide can be administered at 1 hyaluronidase U/therapeutic agent U (1:1) to 50:1 or more, for example, at or about 1:~~1,2~~1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1 or more.

~~[0498]~~(394) The formulations provided herein, including co-formulations and/or stable formulations, can be prepared for single dose administration, multiple dose administration or continuous infusion administrations. Implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained (see e.g., U.S. Pat. No. 3,710,795), is also contemplated herein.

~~[0499]~~(395) For example, formulations of pharmaceutically therapeutically active compounds and derivatives thereof are provided for administration to humans and animals in unit dosage forms or multiple dosage forms. For example, compounds can be formulated as tablets, capsules, pills, powders, granules, sterile parenteral solutions or suspensions, oral solutions or suspensions, or ~~oil-water~~oil—water emulsions containing suitable quantities of the compounds or pharmaceutically acceptable derivatives thereof. Each unit dose contains a predetermined quantity of therapeutically active compound(s) sufficient to produce the desired therapeutic effect, in association with the required pharmaceutical carrier, vehicle or diluent. Examples of unit dose forms include ampoules and syringes and individually packaged tablets or capsules. Unit dose forms can be administered in fractions or multiples thereof. A multiple dose form is a plurality of identical unit dosage forms packaged in a single container to be administered in segregated unit dose forms. Examples of multiple dose forms include vials, bottles of tablets or capsules or bottles of pints or gallons. Hence, multiple dose form is a multiple of unit doses that are not segregated in packaging. Generally, dosage forms or compositions containing active ingredient in the range of 0.005% to 100% with the balance made up from non-toxic carrier can be prepared.

~~[0500]~~(396) Compositions provided herein typically are formulated for administration by subcutaneous route, although other routes of administration are contemplated, such as any route known to those of skill in the art including intramuscular, intraperitoneal, intravenous, intradermal, intralesional, intraperitoneal injection, epidural, vaginal, rectal, local, otic, transdermal administration or any route of administration. Formulations suited for such routes are known to one of skill in the art. Administration can be local, topical or systemic depending upon the locus of treatment. Local administration to an area in need of treatment can be achieved by, for example, but not limited to, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant. Compositions also can be administered with other biologically active agents, either sequentially, intermittently or in the same composition.

~~[0501]~~(397) The most suitable route in any given case depends on a variety of factors, such as the nature of the disease, the tolerance of the subject to a particular administration route, the

severity of the disease, and the particular composition that is used. Typically, the compositions provided herein are administered parenterally. In some examples, modified PH20 polypeptide compositions are administered so that they reach the interstitium of skin or tissues, thereby degrading the interstitial space for subsequent delivery of a therapeutic agent. Thus, in some examples, direct administration under the skin, such as by subcutaneous administration methods, is contemplated. Thus, in one example, local administration can be achieved by injection, such as from a syringe or other article of manufacture containing an injection device such as a needle. In another example, local administration can be achieved by infusion, which can be facilitated by the use of a pump or other similar device. Other modes of administration also are contemplated. For example, modified PH20 polypeptides, included conjugated forms with increased half-life such as PEGylated forms thereof, can be administered intravenously. Pharmaceutical compositions can be formulated in dosage forms appropriate for each route of administration.

[0502](398) Administration methods can be employed to decrease the exposure of selected modified PH20 polypeptides to degradative processes, such as proteolytic degradation and immunological intervention via antigenic and immunogenic responses. Examples of such methods include local administration at the site of treatment. PEGylation of therapeutics increases resistance to proteolysis, increases plasma half-life, and decreases antigenicity and immunogenicity. Examples of PEGylation methodologies are known in the art (see for example, Lu and Felix, Int. J. Peptide Protein Res., 43: 127-138, 1994; Lu and Felix, Peptide Res., 6: 140-6, 1993; Felix et al., Int. J. Peptide Res., 46: 253-64, 1995; Benhar et al., J. Biol. Chem., 269: 13398-404, 1994; Brumeanu et al., J. Immunol., 154: 3088-95, 1995; see also, Caliceti et al. (2003) Adv. Drug Deliv. Rev. 55(10):1261-77 and Molineux (2003) Pharmacotherapy 23 (8 Pt 2):3S-8S). PEGylation also can be used in the delivery of nucleic acid molecules in vivo. For example, PEGylation of adenovirus can increase stability and gene transfer (see, e.g., Cheng et al. (2003) Pharm. Res. 20(9): 1444-51).

[0503](399) Various other delivery systems are known and can be used to administer selected PH20 polypeptides, such as but not limited to, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor mediated endocytosis, and delivery of nucleic acid molecules encoding selected PH20 polypeptides such as retrovirus delivery systems.

[0504](400) Hence, in certain embodiments, liposomes and/or nanoparticles also can be employed with administration of soluble PH20 polypeptides. Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 .mu.mμm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 angstroms containing an aqueous solution in the core.

[0505](401) Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios of lipid to water, liposomes form. Physical characteristics of liposomes depend on the pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel

state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

~~[0506]~~(402) Liposomes interact with cells via different mechanisms: endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. Varying the liposome formulation can alter which mechanism is operative, although more than one can operate at the same time. Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 ~~.mu.m~~µm) should be designed using polymers able to be degraded in vivo. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use herein, and such particles can be easily made.

~~[0507]~~(403) 4. Exemplary PH20-Insulin ~~Co-Formulation~~Co-Formulations

~~[0508]~~(404) Provided herein are stable co-formulations of a fast acting insulin, such as a rapid acting (fast-acting) insulin analog, and a modified PH20 polypeptide. Any of the modified PH20 polypeptides provided herein can be included in a co-formulation with insulin, such as any of the co-formulations described in U.S. application Ser. No. 13/507,263 or 13/507,262 or in International PCT Application Serial No. PCT/US2012/042816.

~~[0509]~~(405) In particular, the modified PH20 polypeptide is a modified PH20 polypeptide that exhibits increased stability under denaturation conditions, such as any set forth in Sections C.1.b. In particular, the PH20 polypeptide is a modified PH20 polypeptide that exhibits increased stability to one or more phenolic preservatives, such as any set forth in Section C.1.b.i. For example, the PH20 polypeptide is a modified PH20 polypeptide that contains an amino acid replacement with P at a position corresponding to position 204 with reference to amino acid positions set forth in SEQ ID NO:3, such as F204P with reference to any of SEQ ID NOS: 3, 7 or 32-66. In other examples, the PH20 polypeptide is a modified PH20 polypeptide that contains an amino acid replacement with R at a position corresponding to position 58 with reference to amino acid positions set forth in SEQ ID NO:3, such as V58R with reference to any of SEQ ID NOS: 3, 7 or 32-66.

~~[0510]~~(406) The fast acting insulin can be a regular insulin or a rapid acting (fast-acting) insulin analog. Insulin is a polypeptide that when processed is composed of 51 amino acids containing an A- and B-chain. Generally, insulin contains an A-chain of about 21 amino acids and a B-chain of about 30 amino acids. The A- and B-chains are linked by disulfide bridges. Exemplary regular insulins include, for example, a human insulin (with an A chain having a sequence of amino acids set forth in SEQ ID NO:862 and a B chain having a sequence of amino acids set forth in SEQ ID NO:863) or a porcine insulin (with an A chain having a sequence of amino acids set forth as amino acid residue positions 88-108 of SEQ ID NO:864 and a B chain having a sequence of amino acids set forth as amino acid residue positions 25-54 of SEQ ID NO:864).

Exemplary fast-acting insulin analogs are insulin variants that contain one or more amino acid modifications compared to a human insulin set forth in SEQ ID NO: 862 and 863 (A and B chains). For example, exemplary insulin analogs are known to one of skill in the art, and include, but are not limited to, glulisine having an A-chain set forth in SEQ ID NO:862 and a B-chain that is a variant of SEQ ID NO:863 (B-chain; LysB3, GluB29), HMR-1 153 having an A-chain set forth in SEQ ID NO:862 and a B-chain that is a variant of SEQ ID NO:863 (B-chain; LysB3, IleB28), insulin aspart having an A-chain set forth in SEQ ID NO:862 and a B-chain that is a variant of SEQ ID NO:863 (B-chain; AspB28), and insulin lispro having an A-chain set forth in SEQ ID NO:862 and a B-chain that is a variant of SEQ ID NO:863 (B-chain; LysB28, ProB29). In every instance above, the nomenclature of the analogs is based on a description of the amino acid substitution at specific positions on the A or B chain of insulin, numbered from the N-terminus of the chain, in which the remainder of the sequence is that of natural human insulin. Exemplary of such analog forms, are set forth in SEQ ID NOS:862 (A-chain) and having a B-chain set forth in any of SEQ ID NOS: 865-867.

~~[0511]~~(407) The co-formulations are stable as a liquid formulation for prolonged periods of time for at least 1 month at temperatures from or from about 2~~.degree.~~° C. to 8~~.degree.~~° C., inclusive, or for at least 3 days at a temperature from or from about 30~~.degree.~~° C. to 42~~.degree.~~° C., inclusive. For example, the co-formulations are stable and retain activity of the PH20 hyaluronidase and insulin at ~~"~~“refrigerator~~"~~” conditions, for example, at 2~~.degree.~~° C. to 8~~.degree.~~° C., such as at or about 4~~.degree.~~° C., for at least at least 2 months, 3 months, 4 months, 5 months, 6 months, or 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23 months, 24 months, 25 months, 26 months, 27 months, 28 months, 29 months or 30 months or more. In another example, the formulations provided herein are stable and retain activity of the PH20 hyaluronidase and insulin at room temperature for example at 18~~.degree.~~° C. to 32~~.degree.~~° C., generally 20~~.degree.~~° C. to 32~~.degree.~~° C., such as 28~~.degree.~~° C. to 32~~.degree.~~° C., for at least 2 weeks to 1 year, for example, at least 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, at least 7 months, at least 8 months, at least 9 months, or at least 1 year or more. In a further example, the formulations provided herein are stable and retain activity of the PH20 hyaluronidase and insulin at elevated temperatures of about or greater than 30~~.degree.~~° C., generally from or from about 30~~.degree.~~° C. to 42~~.degree.~~° C., such as 32~~.degree.~~° C. to 37~~.degree.~~° C. or 35~~.degree.~~° C. to 37~~.degree.~~° C. or about or 37~~.degree.~~° C. for at least 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 35 days, 40 days, 45 days, 50 days, 60 days or more.

~~[0512]~~(408) Assays to assess stability of active agents are well-known to one of skill in the art. Section G provides exemplary assays to assess stability of PH20 hyaluronidase. The stability of insulin can be assessed using similar methods well-known to one of skill in the art. For example, insulin stability and solubility can be assessed by visual assessment (e.g., including changes in color, clarity, presence of aggregates or clumping and material adhesion, or frosting), acid clarification, optical microscopy, reversed phase high performance liquid chromatography (RP-HPLC), in vitro or in vivo bioassays and denaturing and non-denaturing size exclusion chromatography (SEC). In vitro or in vivo bioassays for insulin activity include, but are not limited to, a competitive binding assay using cells expressing insulin receptors (e.g., human

placental cell membranes) and a radiolabeled insulin (see e.g., Weiss et al., (2001) J. Biol. Chem. 276:40018-40024; Duttaroy et al., (2005) Diabetes 54:251-258); insulin-stimulated glucose uptake (Louveau et al., (2004) J Endocrin. 181:271-280, Duttaroy et al., (2005) Diabetes 54:251-258); assays to assess glucose production in the presence of insulin (Wang et al., (2000) J. Biochem., 275:14717-14721, Duttaroy et al., (2005) Diabetes 54:251-258); and studies using diabetic and/or healthy animal models (Atkinson et al., (1999) Nature Med. 5:601-604; Nagoya-Shibata-Yasuda (NSY) mice, Zucker diabetic fatty (ZDF) rats and Gato-Katazaki (GK) rats (Cefalu (2006) ILAR Journal 47:186-198).

~~[0513]~~(409) Examples of such formulations contain 100 U/mL to 1000 U/mL of a modified PH20 polypeptide, and in particular at or about or at least 600 U/mL; 10 U/mL to 1000 U/mL of a fast-acting insulin, and in particular at or at least or about 100 U/mL; NaCl at a concentration of between or about between 80-140 mM; a pH of between or about between 7.0 to 7.8; a buffering agent that maintains the pH range of between or about between 7.0 to 7.8; 0.1% to 0.4% preservative as a mass concentration (w/v). Optionally, a further stabilizing agent can be included. For example, the co-formulations provided herein contain 1 mM to 100 mM of a buffering agent. For example, the co-formulations provided herein contain 0.005% to 0.5% surfactant. Exemplary co-formulations provided herein also can contain less than 60 mM glycerin (glycerol) and 2 mM to or to about 50 mM of an antioxidant.

~~[0514]~~(410) The following stable formulations are exemplary only and provide a platform from which minor adjustments can be made. It is understood that very small changes in the concentrations of the various excipients and other components (e.g., ~~.+-.~~f 15% of the stated concentrations), or small changes in pH, can be made while retaining some if not all of the insulin solubility and stability and PH20 stability. Further changes also can be made by adding or removing excipients. For example, the type of stabilizing surfactant can be changed.

~~[0515]~~(411) For example, the exemplary co-formulations herein contain 100 U/mL to 1000 U/mL of a modified PH20 polypeptide, and in particular at least or about at least or about 600 U/mL of a modified PH20 polypeptide; 10 U/mL to 1000 U/mL of a fast-acting insulin, and in particular at least or about at least or about 100 U/mL of a fast-acting insulin; from or from about 10 mM to or to about 50 mM Tris (e.g., from or from about 20 mM to 40 mM Tris, such as or as about 20 mM, 25 mM, 30 mM, 35 mM or 40 mM Tris); from or from about 80 mM to or to about 160 mM NaCl (e.g., at or about 80 mM, 90 mM, 100 mM, 110 mM 120 mM, 130 mM, 140 mM, 150 mM or 160 mM NaCl); from or from about 2 mM to or to about 50 mM methionine (e.g., at or about 5 mM, 10 mM, 20 mM, 30 mM, 40 mM or 50 mM methionine); from or from about 0 mM to or to about 50 mM glycerin (e.g., at or about 5 mM, 10 mM, 20 mM, 30 mM, 40 mM or 50 mM glycerin); from or from about 0.005% to or to about 0.5% poloxamer 188, such as 0.01% to 0.05% (e.g., at or about 0.01%, 0.02%, 0.03%, 0.04% or 0.05% poloxamer 188); from or from about 0.05% to or to about 0.25% phenol (e.g., at or about 0.1%, 0.12%, 0.125%, 0.13%, 0.14%, 0.15%, 0.16% or 0.17% phenol); and from or from about 0.05% to or to about 0.4% m-cresol (e.g., at or about 0.075%, 0.08%, 0.09%, 0.1%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16% or 0.17% m-cresol). The formulations are prepared with a pH from or from about 7.0 to or to about 7.6 (e.g., at or about pH 7.0, 7.1, 7.2, 7.3, 7.4, 7.5 or 7.6). In further examples, zinc is included at a concentration of or about 0.017 mg/100 U, 0.018 mg/100 U, 0.02 mg/100 U, 0.022 mg/100 U or 0.024 mg/100 U insulin.

[0516](412) In particular examples, the fast acting insulin is insulin aspart, insulin lispro or insulin glulisine. Exemplary co-formulations provided herein that contain a modified PH20 polypeptide and insulin lispro are those that contain from or about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 70 mM to or to about 100 mM NaCl (e.g., at or about 80 mM or 100 mM NaCl); from or from about 10 mM to or to about 30 mM methionine (e.g., at or about 10 mM or 20 mM methionine); from or from about 40 mM to or to about 60 mM glycerin (e.g., at or about 50 mM glycerin); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); from or from about 0.017 mg zinc/100 U insulin to or to about 0.024 mg zinc/100 U insulin (e.g., 0.017 mg zinc/100 U insulin, 0.018 mg/100 U, 0.02 mg/100 U, 0.022 mg/100 U or 0.024 mg zinc/100 U insulin); from or from about 0.08% to or to about 0.17% phenol (e.g., 0.1%, 0.125% or 0.13% phenol); and from or from about 0.07% to or to about 0.17% m-cresol (e.g., 0.075%, 0.08%, 0.13% or 0.15% m-cresol). For example, the co-formulations can contain at or about 0.1%) phenol and 0.015% m-cresol; at or about 0.125% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.075%) m-cresol; at or about 0.13% phenol and 0.08% tri-cresolm-cresol; or at or about 0.17% phenol and 0.13% m-cresol. Such formulations of insulin lispro and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.5 (typically a pH of or about pH 7.2).

[0517](413) Exemplary co-formulations provided herein that contain a modified PH20 polypeptide and insulin aspart are those that contain from or from about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 70 mM to or to about 120 mM NaCl (e.g., at or about 80 mM or 100 mM NaCl); from or from about 2 mM to or to about 30 mM methionine, such as 2 mM to 10 mM or 5 mM to 30 mM methionine (e.g., at or about 5 mM, 10 mM or 20 mM methionine); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); from or from about 0.08% to or to about 0.17% phenol (e.g., 0.1%, 0.125% or 0.13% phenol); and from or from about 0.07% to or to about 0.17% m-cresol (e.g., 0.075%, 0.08%, 0.13% or 0.15% m-cresol). For example, the co-formulations can contain at or about 0.1% phenol and 0.015% m-cresol; at or about 0.125% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.08% m-cresol; or at or about 0.17% phenol and 0.13% m-cresol. Such formulations of insulin aspart and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.6 (typically a pH of or about pH 7.4 or 7.3).

[0518](414) Further exemplary formulations provided herein that contain a modified PH20 polypeptide and insulin aspart are those that do not contain phenol. Such exemplary formulations contain from or from about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 70 mM to or to about 120 mM NaCl (e.g., at or about 80 mM or 100 mM NaCl); from or from about 2 mM to or to about 30 mM methionine, such as 2 mM to 10 mM or 5 mM to 30 mM methionine (e.g., at or about 5 mM, 10 mM or 20 mM methionine); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); and from or from about 0.07% to or to about 0.4% m-cresol, such as from or from about 0.2% to 0.4% m-cresol (e.g., 0.3%, 0.315%, 0.35%, 0.4% m-cresol). Such formulations of insulin aspart and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.6 (typically a pH of or about pH 7.4 or 7.3).

[0519](415) Exemplary co-formulations provided herein that contain a modified PH20 polypeptide and insulin glulisine are those that contain from or from about 25 mM to or to about 35 mM Tris (e.g., at or about 30 mM Tris); from or from about 100 mM to or to about 150 mM NaCl (e.g., at or about 100 mM or 140 mM NaCl); from or from about 10 mM to or to about 30 mM methionine (e.g., at or about 10 mM or 20 mM methionine); from or from about 40 mM to or to about 60 mM glycerin (e.g., at or about 50 mM glycerin); from or from about 0.005% to or to about 0.05% poloxamer 188 (e.g., at or about 0.01% poloxamer 188); from or from about 0.08% to or to about 0.17% phenol (e.g., 0.1%, 0.125% or 0.13% phenol); and from or from about 0.07% to or to about 0.17% m-cresol (e.g., 0.075%, 0.08%, 0.13% or 0.15% m-cresol). For example, the co-formulations can contain at or about 0.1% phenol and 0.015% m-cresol; at or about 0.125% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.075% m-cresol; at or about 0.13% phenol and 0.08% m-cresol; or at or about 0.17% phenol and 0.13% m-cresol. Such formulations of insulin glulisine and a modified PH20 polypeptide are prepared with a pH of or about 7.0 to or to about 7.6 (typically a pH of or about pH 7.4).

[0520](416) 5. Packaging, Articles of Manufacture and Kits

[0521](417) Pharmaceutical compounds of modified PH20 polypeptides, or nucleic acids encoding such polypeptides, or derivatives or variants thereof can be packaged as articles of manufacture containing packaging material, a pharmaceutical composition which is effective for treating a disease or disorder, and a label that indicates that the pharmaceutical composition or therapeutic molecule is to be used for treating the disease or disorder. Combinations of a selected modified PH20 polypeptide, or a derivative or variant thereof and an therapeutic agent also can be packaged in an article of manufacture.

[0522](418) The articles of manufacture provided herein contain packaging materials. Packaging materials for use in packaging pharmaceutical products are well known to those of skill in the art. See, for example, U.S. Pat. Nos. 5,323,907, 5,052,558 and 5,033,252, each of which is incorporated herein in its entirety. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers, pumps, bags, vials, containers, syringes, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment. The articles of manufacture can include a needle or other injection device so as to facilitate administration (e.g., sub-epidermal administration) for local injection purposes. A wide array of formulations of the compounds and compositions provided herein are contemplated including a modified PH20 polypeptide and a therapeutic agent, such as a fast-acting insulin, known to treat a particular disease or disorder. The choice of package depends on the PH20 and/or therapeutic agent, and whether such compositions will be packaged together or separately. In one example, the PH20 can be packaged as a mixture with the therapeutic agent. In another example, the components can be packaged as separate compositions

[0523](419) Modified PH20 polypeptides, therapeutic agents and/or articles of manufacture thereof also can be provided as kits. Kits can include a pharmaceutical composition described herein and an item for administration provided as an article of manufacture. For example a PH20 polypeptide can be supplied with a device for administration, such as a syringe, an inhaler, a dosage cup, a dropper, or an applicator. The compositions can be contained in the item for administration or can be provided separately to be added later. The kit can, optionally, include instructions for application including dosages, dosing regimens and instructions for modes of

administration. Kits also can include a pharmaceutical composition described herein and an item for diagnosis. For example, such kits can include an item for measuring the concentration, amount or activity of the selected protease in a subject.

G. ~~Methods of Assessing PH20 Activity and Stability~~METHODS OF ASSESSING PH20 ACTIVITY AND STABILITY

~~[0524]~~(420) Assays can be used to assess the stability and activity of the PH20 polypeptides provided herein. The assays can be used to assess the hyaluronidase activity of the PH20 polypeptide under particular conditions, temperature, and/or over time. Such assays can be used, for example, to determine the stability of the PH20 polypeptide under specific denaturation conditions, including, but not limited to, elevated temperatures greater than or about or 30~~.degree.~~° C. (e.g., 30~~.degree.~~° C. to 42~~.degree.~~° C. such as or about 37~~.degree.~~° C.), agitation, presence of excipients (e.g., preservative), or low or no NaCl (salt). For example, stability under specific conditions can be monitored by assessing activity, solubility, and stability (e.g., formation of aggregates, etc.) in the absence of exposure to the denaturation condition and then at various time points thereafter in the presence of the condition. Hence, stability can be assessed over time. Stability also can be assessed by comparing any one or more of activity, solubility or aggregation in the presence of one or more denaturation conditions compared to a native, wildtype or reference PH20 polypeptide. The assays also can be used make minor adjustments to the formulations provided herein while retaining the stability of both active agents.

~~[0525]~~(421) 1. Hyaluronidase Activity

~~[0526]~~(422) The activity of a modified PH20 polypeptide can be assessed using methods well known in the art. For example, the USP XXII assay for hyaluronidase determines activity indirectly by measuring the amount of undegraded hyaluronic acid, or hyaluronan, (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37~~.degree.~~° C. (USP XXII-NF XVII (1990) 644-645 United States Pharmacopeia Convention, Inc, Rockville, Md.). A Hyaluronidase Reference Standard (USP) or National Formulary (NF) Standard Hyaluronidase solution can be used in an assay to ascertain the activity, in units, of any hyaluronidase. In one example, activity is measured using a microturbidity assay. This is based on the formation of an insoluble precipitate when hyaluronic acid binds with a reagent that precipitates it, such as acidified serum or cetylpyridinium chloride (CPC). The activity is measured by incubating hyaluronidase with sodium hyaluronate (hyaluronic acid) for a set period of time (e.g., 10 minutes) and then precipitating the undigested sodium hyaluronate with the addition of acidified serum or CPC. The turbidity of the resulting sample is measured at 640 nm after an additional development period. The decrease in turbidity resulting from hyaluronidase activity on the sodium hyaluronate substrate is a measure of hyaluronidase enzymatic activity.

~~[0527]~~(423) In another example, hyaluronidase activity is measured using a microtiter assay in which residual biotinylated hyaluronic acid is measured following incubation with hyaluronidase (see e.g., Frost and Stern (1997) Anal. Biochem. 251:263-269, U.S. Pat. Publication No. 20050260186). The free carboxyl groups on the glucuronic acid residues of hyaluronic acid are biotinylated, and the biotinylated hyaluronic acid substrate is covalently coupled to a microtiter plate. Following incubation with hyaluronidase, the residual biotinylated hyaluronic acid

substrate is detected using an avidin-peroxidase reaction, and compared to that obtained following reaction with hyaluronidase standards of known activity.

[0528](424) Other assays to measure hyaluronidase activity also are known in the art and can be used in the methods herein (see e.g., Delpech et al., (1995) Anal. Biochem. 229:35-41; Takahashi et al., (2003) Anal. Biochem. 322:257-263).

[0529](425) Many hyaluronidase assays have been based upon the measurement of the generation of new reducing N-acetylamino groups (Bonner and Cantey, Clin. Chim. Acta 13:746-752, 1966), or loss of viscosity (De Salegui et al., Arch. Biochem. Biophys. 121:548-554, 1967) or turbidity (Dorfman and Ott, J. Biol. Chem. 172:367, 1948). With purified substrates all of these methods suffice for determination of the presence or absence of endoglycosidase activity.

[0530](426) Substantially purified glycosaminoglycan substrates can also be used in a Gel Shift Assay. Glycosaminoglycans are mixed with recombinant PH20, such as a soluble PH20, to test for endoglycosidase activity that results in a shift in substrate mobility within the gel. Examples of such substrates include, but are not limited to, chondroitin-4 and 6 sulfate, dermatan sulfate, heparan-sulfate, which can be obtained from Sigma Chemical. Human umbilical cord Hyaluronan can be obtained from ICN. For example, each test substrate can be diluted to at or about 0.1 mg/mL in a buffer range from pH 3.5-7.5. In such an exemplary assay, at or about 10 .mu.lµl samples of purified soluble PH20 or conditioned media from PH20 expressing cells can be mixed with at or about 90 .mu.lµl of test substrate in desired buffer and incubated for 3 hours at 37.degree.° C. Following incubation, samples are neutralized with sample buffer (Tris EDTA pH 8.0, Bromophenol Blue and glycerol) followed by electrophoresis. Glycosaminoglycans can be detected using any method known in the art, for example, glycosaminoglycans can be detected by staining the gels using 0.5% Alcian Blue in 3% Glacial Acetic Acid overnight followed by destaining in 7% Glacial Acetic Acid. Degradation is determined by comparison of substrate mobility in the presence and absence of enzyme.

[0531](427) Hyaluronidase activity can also be detected by substrate gel zymography (Guentenhoner et al. (1992) Matrix 12:388-396). In this assay, a sample is applied to an SDS-PAGE gel containing hyaluronic acid and the proteins in the sample separated by electrophoresis. The gel is then incubated in an enzyme assay buffer and subsequently stained to detect the hyaluronic acid in the gel. Hyaluronidase activity is visualized as a cleared zone in the substrate gel.

[0532](428) The ability of a PH20 polypeptide, including a modified PH20 polypeptide provided herein, to act as a spreading or diffusing agent also can be assessed. For example, trypan blue dye can be injected subcutaneously with or without a PH20 polypeptide into the lateral skin on each side of nude mice. The dye area is then measured, such as with a microcaliper, to determine the ability of the PH20 polypeptide to act as a spreading agent (U.S. Pat. Pub. No. 20060104968).

[0533](429) The functional activity of a PH20 polypeptide can be compared and/or normalized to a reference standard using any of these assays. This can be done to determine what a functionally equivalent amount of a PH20 polypeptide is. For example, the ability of a PH20

polypeptide to act as a spreading or diffusing agent can be assessed by injecting it into the lateral skin of mice with trypan blue, and the amount required to achieve the same amount of diffusion as, for example, 100 units of a Hyaluronidase Reference Standard, can be determined. The amount of PH20 polypeptide required is, therefore, functionally equivalent to 100 hyaluronidase units.

[0534](430) 2. Solubility

[0535](431) The solubility of a PH20 polypeptide can be determined by any method known to one of the skill in the art. One method for determining solubility is detergent partitioning. For example, a soluble PH20 polypeptide can be distinguished, for example, by its partitioning into the aqueous phase of a Triton.RTM.® X-114 solution at 37.degree.° C. (Bordier et al., (1981) J. Biol. Chem., 256:1604-1607). Membrane-anchored polypeptides, such as lipid-anchored hyaluronidases, including GPI-anchored hyaluronidases, will partition into the detergent-rich phase, but will partition into the detergent-poor or aqueous phase following treatment with Phospholipase C. Phospholipase C is an enzyme that cleaves the phospho-glycerol bond found in GPI-anchored proteins. Treatment with PLC will cause release of GPI-linked proteins from the outer cell membrane.

[0536](432) 3. Purity, Crystallization or Aggregation

[0537](433) The stability of a PH20 polypeptide provided herein also can be assessed using other methods and assays known in the art. In addition to assessing stability based on hyaluronidase activity, stability can be assessed by visual inspection, percent recovery, protein purity and apparent melting temperature.

[0538](434) For example, protein purity can be measured by reversed phase high performance liquid chromatography (RP-HPLC). Protein purity, as determined by RP-HPLC, is the percent of the main PH20 protein peak present, as compared to all of the protein species present. Thus, RP-HPLC, and similar methods known to one of skill in the art, can assess degradation of the enzyme. Protein purity can be assessed over time. Protein purity also can be assessed in the presence of one or more denaturation conditions and in varying amounts thereof. Percent recovery also can be determined as the relative percentage of the polypeptide under various conditions (denaturation conditions, time of storage, mode of storage such as vessel or container, or other similar parameters that can be altered) as compared to a reference sample. PH20 polypeptide stability also can be determined by measuring the oxidation of the hyaluronidase by RP-HPLC. Percent oxidation is a measure of sum of the peak areas of the major (ox-1) and minor (ox-2) peaks.

[0539](435) In one example, the melting temperature of a PH20 polypeptide, such as a modified PH20 polypeptide, can be determined by measuring the hydrodynamic radius of particles by dynamic light scattering under various conditions (e.g., denaturation conditions or other storage conditions). An increase in particle size and a decrease in the melting temperature indicates denaturation and subsequent aggregation of the hyaluronidase.

[0540](436) Other methods known to one of skill in the art that can be used to determine the stability of the hyaluronidase in the co-formulations provided herein, include polyacrylamide gel

electrophoresis (PAGE), immunoblotting, nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, circular dichroism (CD) and dye-based fluorescence assays.

[0541](437) 4. Pharmacodynamics/Pharmacokinetics

[0542](438) The effect of administration of a PH20 polypeptide, such as a modified PH20 polypeptide, alone or in combination with another therapeutic agent, on the pharmacokinetic and pharmacodynamic properties of any administered agent also can be assessed in vivo using animal models and/or human subjects, such as in the setting of a clinical trial. Pharmacokinetic or pharmacodynamic studies can be performed using animal models or can be performed during studies with patients administered with a PH20 polypeptide or modified PH20 polypeptide.

[0543](439) Animal models include, but are not limited to, mice, rats, rabbits, dogs, guinea pigs and non-human primate models, such as cynomolgus monkeys or rhesus macaques. In some instances, pharmacokinetic or pharmacodynamic studies are performed using healthy animals. In other examples, the studies are performed using animal models of a disease for which therapy with hyaluronan is considered, such as animal models of any hyaluronan-associated disease or disorder, for example a tumor model.

[0544](440) The pharmacokinetic properties of a PH20 polypeptide, such as a modified PH20 polypeptide, can be assessed by measuring such parameters as the maximum (peak) concentration (C.sub.max), the peak time (i.e., when maximum concentration occurs; T.sub.max), the minimum concentration (i.e., the minimum concentration between doses; C.sub.min), the elimination half-life (T.sub.1/2) and area under the curve (i.e., the area under the curve generated by plotting time versus concentration; AUC), following administration. The absolute bioavailability of the hyaluronidase can be determined by comparing the area under the curve of hyaluronidase following subcutaneous delivery (AUC.sub.scAUCsc) with the AUC of hyaluronidase following intravenous delivery (AUC.sub.iv). Absolute bioavailability (F), can be calculated using the formula: F=([AUC].sub.sc.times.×dose.sub.sc)/([AUC].sub.iv.times.×dose.sub.iv). A range of doses and different dosing frequency of dosing can be administered in the pharmacokinetic studies to assess the effect of increasing or decreasing concentrations enzyme, such as modified PH20 polypeptide, in the dose.

H. Methods of Treatment and Combination TherapyMETHODS OF TREATMENT AND COMBINATION THERAPY

[0545](441) Provided herein are methods and uses of any of the modified PH20 polypeptides provided herein that exhibit hyaluronidase activity based on its ability to degrade glycosaminoglycan(s) such as hyaluronan. Due to such activity, the modified PH20 polypeptides can be used as a spreading factor to increase the delivery and/or bioavailability of subcutaneously administered therapeutic agents. Delivery of any therapeutic agent, including but not limited to, peptides, proteins, small molecule drugs, nucleic acids, or viruses can be facilitated or enhanced by co-administration with a modified PH20 polypeptide provided herein. For example, modified PH20 polypeptides can be used to increase the delivery of therapeutic agents such as antibodies (e.g., monoclonal antibodies), cytokines, Immune Globulin, an Insulin, or coagulation factors, to a desired locus, such as by increasing penetration of chemotherapeutic

agents into solid tumors. The modified PH20 polypeptides also can be used to treat a hyaluronan-disease or disorder that is characterized by an excess or accumulation of hyaluronan. For example, modified PH20 polypeptides provided herein can be used to for treating a tumor; for treating glycosaminoglycan accumulation in the brain; for treating a cardiovascular disorder; for treating an ophthalmic disorder; for treating pulmonary disease; for treating cellulite; and/or for treating a proliferative disorder.

~~[0546]~~(442) Other methods and uses of a modified PH20 polypeptide include any that are known to one of skill in the art. For example, various forms of PH20 hyaluronidases have been prepared and approved for therapeutic use in humans. For example, animal-derived hyaluronidase preparations include Vitrase~~.RTM.~~® (ISTA Pharmaceuticals), a purified ovine testicular hyaluronidase, and Amphadase~~.RTM.~~® (Amphastar Pharmaceuticals), a bovine testicular hyaluronidase. Hylenex~~.RTM.~~® (Halozyme Therapeutics) is a human recombinant hyaluronidase produced by genetically engineered Chinese Hamster Ovary (CHO) cells containing nucleic acid encoding for soluble rHuPH20 (see e.g., U.S. Pat. No. 7,767,429). Approved therapeutic uses for hyaluronidases include use as an adjuvant to increase the absorption and dispersion of other therapeutic agents for hypodermoclysis (subcutaneous fluid administration), and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents. In addition to these indications, hyaluronidases can be used as a therapeutic or cosmetic agent for the treatment of additional diseases and conditions. For example, hyaluronidase is commonly used, for example, for peribulbar block in local anesthesia prior ophthalmic surgery. The presence of the enzyme prevents the need for additional blocks and reduces the time to the onset of akinesia (loss of eye movement). Peribulbar and sub-Tenon's block are the most common applications of hyaluronidase for ophthalmic procedures. Hyaluronidase also can promote akinesia in cosmetic surgery, such as blepharoplasties and face lifts. It is understood that soluble PH20 hyaluronidases provided herein, including esPH20 hyaluronidases, can be used in any method of treatment or combination therapy for which a PH20 hyaluronidase is used (see e.g., U.S. Publication Nos. US20040268425; US20050260186; US20060104968; and U.S. application Ser. No. 12/381,844, published as U.S. Publication No. ~~US20100074885~~U520100074885; Ser. No. 12/386,249, published as U.S. Publication No. US20090311237; Ser. No. 12/387,225, published as U.S. Publication No. US20090304665; and Ser. No. 12/386,222, published as U.S. Publication No. US2010003238, each incorporated by reference in their ~~entirely~~entirety).

~~[0547]~~(443) Exemplary, non-limiting, methods and uses are described in the following subsections.

~~[0548]~~(444) 1. Methods of Delivering Therapeutic Agents

~~[0549]~~(445) As noted above, hyaluronidase is a spreading or diffusing substance that modifies the permeability of connective tissue through the hydrolysis of hyaluronic acid, a polysaccharide found in the intercellular ground substance of connective tissue, and of certain specialized tissues, such as the umbilical cord and vitreous humor. When no spreading factor is present, materials injected subcutaneously, such as drugs, proteins, peptides and nucleic acid, spread very slowly. Co-injection with hyaluronidase, however, can cause rapid spreading. The rate of diffusion is proportional to the amount of enzyme, and the extent of diffusion is proportional to the volume of solution.

[0550](446) Modified PH20 polypeptides provided herein can be used to promote or enhance the delivery agents and molecules to any of a variety of mammalian tissues in vivo. It can be used to facilitate the diffusion and, therefore, promote the delivery, of small molecule pharmacologic agents as well as larger molecule pharmacologic agents, such as proteins, nucleic acids and ribonucleic acids, and macromolecular compositions than can contain a combination of components including, but not limited to, nucleic acids, proteins, carbohydrates, lipids, lipid-based molecules and drugs (see e.g., U.S. Publication Nos. US20040268425; US20050260186; and US20060104968). Modified PH20 polypeptides can be co-administered and/or co-formulated with a therapeutic agent to improve the bioavailability as well as pharmacokinetic (PK) and/or pharmacodynamic (PD) characteristics of co-formulated or co-administered agents. PK/PD parameters that can be improved by using soluble PH20, such as esPH20, include such measures as C.sub.max (the maximal concentration of agent achieved following absorption in, e.g., the bloodstream), T.sub.max (the time required to achieve maximal concentration), T.sub.1/2 (the time required for the concentration to fall by half), C.sub.min (the minimal concentration of agent following metabolism and excretion), AUC (area under the curve of concentration versus time, a measure of the overall amount of bioavailability), concentrations in various tissues of interest (including, e.g., the rate of achieving desired concentrations, the overall levels, and the duration of maintaining desired levels), and E.sub.max (the maximal effect achieved).

[0551](447) The methods of treatment provided herein include combination therapies with a therapeutic agent for the treatment of a disease or disorder for which the therapeutic agent threats. Any therapeutic agent that ameliorates and or otherwise lessens the severity of a disease or condition can be combined with a modified PH20 polypeptide provided herein in order to increase the bioavailability of such therapeutic agent. In particular, modified PH20 polypeptides provided herein can be used in each and all of the combinations described in applications see e.g., U.S. Publication Nos. US20040268425; US20050260186; US20060104968 and U.S. application Ser. No. 12/381,844, published as U.S. Publication No. US20100074885; Ser. No. 12/386,249, published as U.S. Publication No. US20090311237; Ser. No. 12/387,225, published as U.S. Publication No. US20090304665; and Ser. No. 12/386,222, published as U.S. Publication No. US2010003238 in place of the disclosed hyaluronidase or hyaluronidase degrading enzyme.

[0552](448) Modified PH20 polypeptides can be administered prior to, subsequent to, intermittently with or simultaneously with the therapeutic agent preparation. Generally, the modified PH20 polypeptide is administered prior to or simultaneously with administration of the therapeutic agent preparation to permit the PH20 to degrade the hyaluronic acid in the interstitial space. The PH20 can be administered at a site different from the site of administration of the therapeutic molecule or the soluble PH20 can be administered at a site the same as the site of administration of the therapeutic molecule.

[0553](449) Examples of pharmaceutical, therapeutic and cosmetic agents and molecules that can be administered with hyaluronidase include, but are not limited to, a chemotherapeutic or anticancer agent, an analgesic agent, an antibiotic agent, an anti-inflammatory agent, an antimicrobial agent, an amoebicidal agent, a trichomonacidal agent, an anti-parkinson agent, an anti-malarial agent, an anticonvulsant agent, an anti-depressant agent, an anti-arthritic agent, an anti-fungal agent, an antihypertensive agent, an antipyretic agent, an anti-parasitic agent, an

antihistamine agent, an alpha-adrenergic agonist agent, an alpha blocker agent, an anesthetic agent, a bronchial dilator agent, a biocide agent, a bactericide agent, a bacteriostatic agent, a beta adrenergic blocker agent, a calcium channel blocker agent, a cardiovascular drug agent, a contraceptive agent, a cosmetic or esthetic agent, a decongestant agent, a diuretic agent, a depressant agent, a diagnostic agent, an electrolyte agent, a hypnotic agent, a hormone agent, a hyperglycemic agent, a muscle relaxant agent, a muscle contractant agent, an ophthalmic agent, a parasympathomimetic agent, a psychic energizer agent, a sedative agent, a sleep inducer, a sympathomimetic agent, a tranquilizer agent, a urinary agent, a vaginal agent, a viricide agent, a vitamin agent, a non-steroidal anti-inflammatory agent, or an angiotensin converting enzyme inhibitor agent, and any combination thereof. In particular, therapeutic agents include antibodies, including monoclonal antibodies, bisphosphonates, insulins, coagulation factors, cytokines and Immun Globulins.

[0554](450) For example, modified PH20 polypeptides provided herein can be used to increase the delivery of chemotherapeutic agents. Hyaluronidases have also been used to enhance the activity of chemotherapeutics and/or the accessibility of tumors to chemotherapeutics (Schuller et al., 1991, Proc. Amer. Assoc. Cancer Res. 32:173, abstract no. 1034; Czejka et al., 1990, Pharmazie 45:H9H.9; Baumgartner et al. (1988) Reg. Cancer Treat. 1:55-58; Zanker et al. (1986) Proc. Amer. Assoc. Cancer Res. 27:390). Combination chemotherapy with hyaluronidase is effective in the treatment of a variety of cancers including urinary bladder cancer (Horn et al.:, 1985, J. Surg. Oncol. 28:304-307), squamous cell carcinoma (Kohno et al., 94, J. Cancer Res. Oncol. 120:293-297), breast cancer (Beckenlehner et al., 1992, J. Cancer Res. Oncol. 118:591-596), and gastrointestinal cancer (Scheithauer et al., 1988, Anticancer Res. 8:391-396). In this example, the modified PH20 hyaluronidase enhances penetration of chemotherapeutic or other anti-cancer agents into solid tumors, thereby treating the disease.

[0555](451) Compositions containing soluble PH20 can be injected intratumorally with anti-cancer agents or intravenously for disseminated cancers or hard to reach tumors. The anticancer agent can be a chemotherapeutic, an antibody, a peptide, or a gene therapy vector, virus or DNA. Additionally, hyaluronidase can be used to recruit tumor cells into the cycling pool for sensitization in previously chemorefractory tumors that have acquired multiple drug resistance (St Croix et al., (1998) Cancer Lett September 131(1): 35-44).

[0556](452) Exemplary anti-cancer agents that can be administered after, coincident with or before administration of a soluble PH20, such as an esPH20, include, but are not limited to Acivicins; Aclarubicins; Acodazoles; Acronines; Adozelesins; Aldesleukins; Alemtuzumabs; Alitretinoins (9-Cis-Retinoic Acids); Allopurinols; Altretamines; Alvocidibs; Ambazones; Ambomycins; Ametantrones; Amifostines; Aminoglutethimides; Amsacrines; Anastrozoles; Anaxirones; Ancitabines; Anthramycins; Apaziquones; Argimesnas; Arsenic Trioxides; Asparaginases; Asperlins; Atrimustines; Azacitidines; Azetepas; Azotomycins; Banoxantrones; Batabulins; Batimastats; BCG Live; Benaxibines; Bendamustines; Benzodepas; Bexarotenes; Bevacizumab; Bicalutamides; Bietaserpines; Biricodars; Bisantrenes; Bisantrenes; Bisnafide Dimesylates; Bizelesins; Bleomycins; Bortezomibs; Brequinars; Bropirimines; Budotitanes; Busulfans; Cactinomycins; Calusterones; Canertinibs; Capecitabines; Caracemides; Carbetimers; Carboplatins; Carboquones; Carmofurs; Carmustines with Polifeprosans; Carmustines; Carubicins; Carzelesins; Cedefingols; Celecoxibs; Cemadotins; Chlorambucils; Cioteronels; Ciplactin; Cirolemycins; Cisplatins; Cladribines; Clanfenurs; Clofarabines; Crisnatols;

Cyclophosphamides; Cytarabine liposomals; Cytarabines; Dacarbazines; Dactinomycins; Darbepoetin Alfas; Daunorubicin liposomals; Daunorubicins/Daunomycins; Daunorubicins; Decitabines; Denileukin Diftitoxes; Dexniguldipines; Dexonas; Dexrazoxanes; Dezaguanines; Diaziquones; Dibrospidiums; Dienogests; Dinalins; Disermolides; Docetaxels; Dofequidars; Doxifluridines; Doxorubicin liposomals; Doxorubicin HCL; Doxorubicin HCL liposome injection; Doxorubicins; Droloxifenes; Dromostanolone Propionates; Duazomycins; Ecomustines; Edatrexates; Edotecarins; Eflornithines; Elacridars; Elinafides; Elliott's B Solutions; Elsamitrucins; Emitefurs; Enloplatins; Enpromates; Enzastaurins; Epipropidines; Epirubicins; Epoetin alfas; Eptaloprosts; Erbulozoles; Esorubicins; Estramustines; Etanidazoles; Etoglucids; Etoposide phosphates; Etoposide VP-16s; Etoposides; Etoprines; Exemestanes; Exisulinds; Fadrozoles; Fazarabines; Fenretinides; Filgrastims; Floxuridines; Fludarabines; Fluorouracils; 5-fluorouracils; Fluoxymesterones; ~~Fluorocitabines~~Flurocitabines; Fosquidones; Fostriecins; Fostriecins; Fotretamines; Fulvestrants; Galarubicins; Galocitabines; Gemcitabines; Gemtuzumabs/Ozogamicins; Geroquinols; Gimatecans; Gimeracils; Gloxazones; Glufosfamides; Goserelin acetates; Hydroxyureas; Ibritumomabs/Tiuxetans; Idarubicins; Ifosfamides; Ilmofosines; Ilomastats; Imatinib mesylates; Imexons; Improsulfans; Indisulams; Inproquones; Interferon alfa~~-2 as~~-2as; Interferon alfa-2bs; Interferon Alfas; Interferon Betas; Interferon Gammas; Interferons; Interleukin-2s and other ~~Lnterleukins~~Interleukins (including recombinant Interleukins); Intoplicines; Iobenguanes [131-I]; Iproplatins; Irinotecans; Irsogladines; Ixabepilones; Ketotrexates; L-Alanosines; Lanreotides; Lapatinibs; Ledoxantrones; Letrozoles; Leucovorins; Leuprolides; Leuprorelins (Leuprolides); Levamisoles; Lexacalcitols; Liarozoles; Lobaplatins; Lometrexols; Lomustines/CCNUs; Lomustines; Lonafarnibs; Losoxantrones; Lurtotecans; Mafosfamides; Mannosulfans; Marimastats; Masoprocols; Maytansines; Mechlorethamines; Mechlorethamines/Nitrogen mustards; Megestrol acetates; Megestrols; Melengestrols; Melphalans; Melphalan L-PAMs; Menogarils; Mepitiostanes; Mercaptopurines; 6-~~Mercaptopurine~~Mecaptopurine; Mesnas; Metesinds; Methotrexates; Methoxsalens; Metomidates; Metoprines; Meturedepas; Miboplatins; Miproxifenes; Misonidazoles; Mitindomides; Mitocarcins; Mitocromins; Mitoflaxones; Mitogillins; Mitoguazones; Mitomalcins; Mitomycin Cs; Mitomycins; Mitonafides; Mitoquidones; Mitospers; Mitotanes; Mitoxantrones; Mitozolomides; Mivobulins; Mizoribines; Mofarotenes; Mopidamols; Mubritinibs; Mycophenolic Acids; Nandrolone Phenpropionates; Nedaplatins; Nelarabines; Nemorubicins; Nitracrines; Nocodazoles; Nofetumomabs; Nogalamycins; Nolatrexeds; Nortopixantrones; Octreotides; Oprelvekins; Ormaplatins; Ortataxels; Oteracils; Oxaliplatins; Oxisurans; Oxophenarsines; Paclitaxels; Pamidronates; Patupilones; Pegademases; Pegaspargases; Pegfilgrastims; Peldesines; Peliomycins; Pelitrexols; Pemetrexeds; Pentamustines; Pentostatins; Peplomycins; Perfosfamides; Perifosines; Picoplatins; Pinafides; Pipobromans; Piposulfans; Pirfenidones; Piroxantrones; Pixantrones; Plevitrexeds; Plicamycin Mithramycins; Plicamycins; Plomestanes; Plomestanes; Porfimer sodiums; Porfimers; Porfiromycins; Prednimustines; Procarbazines; Propamidines; Prospidiums; Pumitepas; Puromycins; Pyrazofurins; Quinacrines; Ranimustines; Rasburicases; Riboprines; Ritrosulfans; Rituximabs; Rogletimides; Roquinimexs; Rufocromomycins; Sabarubicins; Safingols; Sargramostims; Satraplatins; Sebriplatins; Semustines; Simtrazenes; Sizofirans; Sobuzoxanes; Sorafenibs; Sparfosates; Sparfosic Acids; Sparsomycins; Spirogermaniums; Spiromustines; Spiroplatins; Spiroplatins; Squalamines; Streptonigrins; Streptovarycins; Streptozocins; Sufosfamides; Sulofenurs; Sunitinib Malate; 6-TG; Tacedinalines; Talcs; Talisomycins; Tallimustines; Tamoxifens; Tariquidars; Tauromustines; Tecogalans; Tegafurs; Teloxantrones;

Temoporfins; Temozolomides; Teniposides/VM-26s; Teniposides; Teroxirones; Testolactones; Thiamiprines; Thioguanines; Thiotepas; Tiamiprines; Tiazofurins; Tilomisoles; Tilorones; Timcodars; Timonacics; Tirapazamines; Topixantrones; Topotecans; Toremifenes; Tositumomabs; Trabectedins (Ecteinascidin 743); Trastuzumabs; Trestolones; Tretinoins/ATRA; Triciribines; Trilostanes; Trimetrexates; Triplatin Tetranitrates; Triptorelins; Trofosfamides; Tubulozoles; Ubenimexs; Uracil Mustards; Uredepas; Valrubicins; Valspodars; Vapreotides; Verteporfins; Vinblastines; Vincristines; Vindesines; Vinepidines; Vinflunines; Vinformides; Vinglycinates; Vinleucinols; Vinleurosines; Vinorelbines; Vinrosidines; Vintriptols; Vinzolidines; Vorozoles; Xanthomycin A's (Guamecyclines); Zeniplatins; Zilascorbs [2-H]; Zinostatins; Zoledronate; Zorubicins; and Zosuquidars, for example:

~~[0557]~~(453) Aldesleukins (e.g., PROLEUKIN~~.RTM.~~®); Alemtuzumabs (e.g., CAMPATH~~.RTM.~~®); Alitretinoins (e.g., PANRETIN~~.RTM.~~®); Allopurinols (e.g., ZYLOPRIM~~.RTM.~~®); Altretamines (e.g., HEXALEN~~.RTM.~~®); Amifostines (e.g., ETHYOL~~.RTM.~~®); Anastrozoles (e.g., ARIMIDEX~~.RTM.~~®); Arsenic Trioxides (e.g., TRISENOX~~.RTM.~~®); Asparaginases (e.g., ELSPAR~~.RTM.~~®); BCG Live (e.g., TICE~~.RTM.~~® BCG); Bexarotenes (e.g., TARGRETIN~~.RTM.~~®); Bevacizumab (~~AVASTDM.RTM.~~AVASTIN®); Bleomycins (e.g., BLENOXANE~~.RTM.~~®); Busulfan intravenous (e.g., BUSULFEX~~.RTM.~~®); Busulfan orals (e.g., MYLERAN~~.TM.~~™); Calusterones (e.g., METHOSARB~~.RTM.~~®); Capecitabines (e.g., XELODA~~.RTM.~~®); Carboplatins (e.g., PARAPLATIN~~.RTM.~~®); Carmustines (e.g., BCNU~~.RTM.~~®, BiCNU~~.RTM.);~~®); Carmustines with Polifeprosans (e.g., GLIADEL~~.RTM.~~® Wafer); Celecoxibs (e.g., CELEBREX~~.RTM.~~®); Chlorambucils (e.g., LEUKERAN~~.RTM.~~®); Cisplatins (e.g., PLATINOL~~.RTM.~~®); Cladribines (e.g., ~~LEU-STATIN.RTM.~~LEUSTATIN®, 2-CdA~~.RTM.~~®); Cyclophosphamides (e.g., CYTOXAN~~.RTM.~~®, NEOSAR~~.RTM.~~®); Cytarabines (e.g., CYTOSAR-U~~.RTM.~~®); Cytarabine liposomals (e.g., DepoCyt~~.RTM.~~®); Dacarbazines (e.g., ~~DTIC-Dome®~~DTIC-Domeu): Dactinomycins (e.g., COSMEGEN~~.RTM.~~®); Darbepoetin Alfas (e.g., ARANESP~~.RTM.~~®); Daunorubicin liposomals (e.g. DAUNOXOME~~.RTM.~~®); Daunorubicins/Daunomycins (e.g., CERUBIDINE~~.RTM.~~®); Denileukin Diftitoxes (e.g., ONTAK~~.RTM.~~®); Dexrazoxanes (e.g., ZINECARD~~.RTM.~~®); Docetaxels (e.g., TAXOTERE~~.RTM.~~®); Doxorubicins (e.g., ADRIAMYCIN~~.RTM.~~®, RUBEX~~.RTM.~~®); Doxorubicin liposomals, including Doxorubicin HCL liposome injections (e.g., DOXIL~~.RTM.~~®); Dromostanolone propionates (e.g., DROMOSTANOLONE~~.RTM.~~® and MASTERONE~~.RTM.~~® Injection); Elliott's B Solutions (e.g., Elliott's B Solution~~.RTM.~~®); Epirubicins (e.g., ELLENCE~~.RTM.~~®); Epoetin alfas (e.g., EPOGEN~~.RTM.~~®); Estramustines (e.g., EMCYT~~.RTM.~~®); Etoposide phosphates (e.g., ETOPOPHOS~~.RTM.~~®); Etoposide VP-16s (e.g., VEPESID~~.RTM.~~®); Exemestanes (e.g., AROMASIN~~.RTM.~~®); Filgrastims (e.g., NEUPOGEN~~.RTM.~~®); Floxuridines (e.g., FUDR~~.RTM.~~®); Fludarabines (e.g., FLUDARA~~.RTM.~~®); Fluorouracils incl. 5-FUs (e.g., ADRUCIL~~.RTM.~~®); Fulvestrants (e.g., FASLODEX~~.RTM.~~®); Gemcitabines (e.g., GEMZAR~~.RTM.~~®); Gemtuzumabs/Ozogamicins (e.g., MYLOTARG~~.RTM.~~®); Goserelin acetates (e.g., ZOLADEX~~.RTM.~~®); Hydroxyureas (e.g., HYDREA~~.RTM.~~®); Ibritumomabs/Tiuxetans (e.g., ZEVALIN~~.RTM.~~®); Idarubicins (e.g., IDAMYCIN~~.RTM.~~®); Ifosfamides (e.g., IFEX~~.RTM.~~®); Imatinib mesylates (e.g., GLEEVEC~~.RTM.~~®); Interferon alfa~~-2 as~~-2as (e.g., ROFERON-A~~.RTM.~~®); Interferon alfa-2bs (e.g., INTRON A~~.RTM.~~®); Irinotecans (e.g., CAMPTOSAR~~.RTM.~~®); Letrozoles (e.g., FEMARA~~.RTM.~~®); Leucovorins (e.g., WELLCOVORIN~~.RTM.~~®, LEUCOVORIN~~.RTM.~~®); Levamisoles (e.g., ERGAMISOL~~.RTM.~~®); Lomustines/CCNUs (e.g., CeeNU~~.RTM.~~®);

Mechlorethamines/Nitrogen mustards (e.g., MUSTARGEN.RTM.®); Megestrol acetates (e.g., MEGACE.RTM.®); Melphalans/L-PAMs (e.g., ALKERAN.RTM.®); Mercaptopurine incl. 6-MPs (e.g., PURINETHOL.RTM.®); Mesnas (e.g., MESNEX.RTM.®); Methotrexates; Methoxsalens (e.g., UVADEX.RTM.®); Mitomycin Cs (e.g., MUTAMYCIN.RTM.®, MITOZYTREX.RTM.®); Mitotanes (e.g., LYSODREN.RTM.®); Mitoxantrones (e.g., NOVANTRONE.RTM.®); Nandrolone Phenpropionates (e.g., DURABOLIN-50.RTM. ®); Nofetumomabs (e.g., VERLUMA.RTM.®); Oprelvekins (e.g., NEUMEGA.RTM.®); Oxaliplatins (e.g., ELOXATIN.RTM.®); Paclitaxels (e.g., PAXENE.RTM.®, TAXOL.RTM.®); Pamidronates (e.g., AREDIA.RTM.®); Pegademases (e.g., ADAGEN.RTM.®); Pegaspargases (e.g., ONCASPAR.RTM.®); Pegfilgrastims (e.g., NEULASTA.RTM.®); Pentostatins (e.g., NIPENT.RTM.®); Pipobromans (e.g., VERCYTE.RTM.®); Plicamycin/Mithramycins (e.g., MITHRACIN.RTM.®); Porfimer sodiums (e.g., PHOTOFRTN.RTM.PHOTOFRIN®); Procarbazines (e.g., MATULANE.RTM.®); Quinacrines (e.g., ATABRTNE.RTM.ATABRINE®); Rasburicases (e.g., ELITEK.RTM.®); Rituximabs (e.g., RITUXAN.RTM.®); Sargramostims (e.g., PROKINE.RTM.®); Streptozocins (e.g., ZANOSAR.RTM.®); Sunitinib Malates (e.g., SUTENT.RTM.®); Talcs (e.g., SCLEROSOL.RTM.®); Tamoxifens (e.g., NOLVADEX.RTM.®); Temozolomides (e.g., TEMODAR.RTM.®); Teniposides/VM-26s (e.g., VUMON.RTM.®); Testolactones (e.g., TESLAC.RTM.®); Thioguanines incl. 6-TG; Thiotepas (e.g., THIOPLEX.RTM.®); Topotecans (e.g., HYCAMTEN.RTM.HYCAMTIN®); Toremifenes (e.g., FARESTON.RTM.®); Tositumomabs (e.g., BEXXAR.RTM.®); Trastuzumabs (e.g., HERCEPTIN.RTM.®); Tretinoins/ATRA (e.g., VESANOID.RTM.®); Uracil Mustards; Valrubicins (e.g., VALSTAR.RTM.®); Vinblastines (e.g., VELBAN.RTM.®); Vincristines (e.g., ONCOVIN.RTM.®); Vinorelbines (e.g., NAVELBINE.RTM.®); and Zoledronates (e.g., ZOMETA.RTM.®).

[0558](454) For example, exemplary antibiotic agents include, but are not limited to, Aminoglycosides; Amphenicols; Ansamycins; Carbacephems; Carbapenems; Cephalosporins or Cephems; Cephamycins; Clavams; Cyclic lipopeptides; Diaminopyrimidines; Ketolides; Lincosamides; Macrolides; Monobactams; Nitrofurans; Oxacephems; Oxazolidinones; Penems, thienamycins and miscellaneous beta-lactams; Penicillins; Polypeptides antibiotics; Quinolones; Sulfonamides; Sulfones; Tetracyclines; and other antibiotics (such as Clofoctols, Fusidic acids, Hexedines, Methenamines, Nitrofurantoins Nitroxolines, Ritipenems, Taurolidines, Xibomols).

[0559](455) Also included among exemplary therapeutic agents are coagulation factors or other blood modifiers such as antihemophilic factors, anti-inhibitor coagulant complexes, antithrombin III, coagulation Factor V, coagulation Factor VIII, coagulation Factor IX, plasma protein fractions, von Willebrand factors; antiplatelet agents (including, for example, abciximabs, anagrelides, cilostazols, clopidogrel bisulfates, dipyridamoles, epoprostenols, eptifibatides, tirofibans; colony stimulating factors (CSFs) (including, for example, Granulocyte CSFs and Granulocyte Macrophage CSFs); erythropoiesis stimulators (including, for example, erythropoietins such as darbepoetin alfas) and epoetin alfas; hemostatics and albumins (including, for example, aprotinins, combinations of antihemophilic factors and plasma, Desmopressin Acetates, and albumins); immune globulins, as well as hepatitis B immune globulins; thrombin inhibitors (including for example direct thrombin inhibitors and lepirudin), and drotrecogin alfas; anticoagulants (including, for example, dalteparins, enoxaparins and other heparins, and warfarins).

[0560](456) Exemplary antibodies or other therapeutic agents include, but are not limited to, Cetuximab (IMC-C225; Erbitux.RTM.®); Trastuzumab (Herceptin.RTM.®); Rituximab (Rituxan.RTM.®; MabThera.RTM.®); Bevacizumab (Avastin.RTM.®); Alemtuzumab (Campath.RTM.®; Campath-1H.RTM.®; Mabcampath.RTM.®); Panitumumab (ABX-EGF; Vectibix.RTM.®); Ranibizumab (Lucentis.RTM.®); Ibritumomab; Ibritumomab tiuxetan (Zevalin.RTM.®); Tositumomab; Iodine I 131 Tositumomab (BEXXAR.RTM.®); Catumaxomab (Removab.RTM.®); Gemtuzumab; Gemtuzumab ozogamicin (Mylotarg.RTM.®); Abatacept (CTLA4-Ig; Orencia.RTM.®); Belatacept (L104EA29YIg; LEA29Y; LEA); Ipilimumab (MDX-010; MDX-101); Tremelimumab (ticilimumab; CP-675,206); PRS-010 (see e.g., US20090042785); PRS-050 (US7585940U.S. Pat. No. 7,585,940; US20090305982); Aflibercept (VEGF Trap, AVE005; Holash et al., (2002) PNAS 99:11393-11398); Volociximab (M200); F200 (Chimeric (human/murine) IgG4 Fab fragment of Volociximab (M200)); MORAb-009 Mouse/human chimeric IgG1 (US20050054048); Soluble fusion protein:Anti-mesothelin Fv linked to atruncatedda truncated Pseudomonas exotoxin A (SS1P (CAT-5001); US20070189962); Cixutumumab (DVIC-A12IMC-A12); Nimotuzumab (h-R3) (Spicer (2005) Curr Opin Mol Ther 7:182-191); Zalutumumab (HuMax-EGFR; Lammerts van Bueren et al. (2008) PNAS 105:6109-14); Necitumumab IMC-11F8 (Li et al. (2008) Structure 16:216-227); Sym004 (Pedersen et al. 2010 Cancer Res 70:588-597); and mAb-425.

[0561](457) In particular, therapeutic agents include, but are not limited to, immunoglobulins, Interferon beta, Interferon alpha-2 as-2as, Interferon alpha-1s, Interferon alpha-n3salpha-n3 s, Interferon beta-1, Interferon beta-1as, Interferon gamma-1bs, Peg-interferon alpha-2 and Peginterferon alpha-2bs, insulin, a bisphosphate (e.g., Pamidronates or Zoledronates), Docetaxels, Doxorubicins, Doxorubicin liposomals and bevacizumabs.

[0562](458) Other exemplary therapeutic agents that can be combined by co-administration and/or co-formulation with a modified PH20 polypeptide provided herein, include, but are not limited to, Adalimumabs, Agalsidase Betas, Alefacepts, Ampicillins, Anakinras, Antipoliomyelitic Vaccines, Anti-Thymocytes, Azithromycins, Becaplermins, Caspofungins, Cefazolins, Cefepimes, Cefotetans, Ceftazidimes, Ceftriaxones, Cetuximabs, Cilastatins, Clavulanic Acids, Clindamycins, Darbepoetin Alfas, Daclizumabs, Diphtheria, Diphtheria antitoxins, Diphtheria Toxoids, Efalizumabs, Epinephrines, Erythropoietin Alphas, Etanercepts, Filgrastims, Fluconazoles, Follicle-Stimulating Hormones, Follitropin Alphas, Follitropin Betas, FosphenyloinsFosphenytoins, Gadodiamides, Gadopentetates, Gatifloxacins, Glatiramers, GM-CSF's, Goserelins, Goserelin acetates, Granisetrons, Haemophilus Influenza B's, Haloperidols, Hepatitis vaccines, Hepatitis A Vaccines, Hepatitis B Vaccines, Ibritumomab Tiuxetans, Ibritumomabs, Tiuxetans, Immunoglobulins, Hemophilus influenza vaccines, Influenza Virus Vaccines, Infliximabs, Insulins, Insulin Glargines, Interferons, Interferon alphas, Interferon Betas, Interferon Gammas, Interferon alpha-2a's, Interferon alpha-2b's, Interferon alpha-1's, Interferon alpha-n3's, Interferon Betas, Interferon Beta-1a's, Interferon Gammas, Interferon alpha-consensus, Iodixanols, IohexylsIohexols, Iopamidols, Ioversols, Ketorolacs, Laronidases, Levofloxacins, Lidocaines, Linezolids, Lorazepams, Measles Vaccines, Measles virus, Mumps viruses, Measles-Mumps-Rubella Virus Vaccines, Rubella vaccines, Medroxyprogesterones, Meropenems, Methylprednisolones, Midazolams, Morphines, Octreotides, Omalizumabs, Ondansetrons, Palivizumabs, Pantoprazoles, Pegaspargases, Pegfilgrastims, Peg-Interferon Alfa-2a's, Peg-Interferon Alfa-2b's, Pegvisomants, Pertussis vaccines, Piperacillins, Pneumococcal Vaccines and Pneumococcal Conjugate Vaccines, Promethazines, Reteplases, Somatropins,

Sulbactams, Sumatriptans, Tazobactams, Tenecteplases, Tetanus Purified Toxoids, Ticarcillins, Tositumomabs, Triamcinolones, Triamcinolone Acetonides, Triamcinolone hexacetonides, Vancomycins, Varicella Zoster immunoglobulins, Varicella vaccines, other vaccines, Alemtuzumabs, Alitretinoins, Allopurinols, Altretamines, Amifostines, Anastrozoles, Arsenics, Arsenic Trioxides, Asparaginases, Bacillus Calmette-Guerin (BCG) vaccines, BCG Live, Bexarotenes, Bleomycins, Busulfans, Busulfan intravenous, Busulfan orals, Calusterones, Capecitabines, Carboplatins, Carmustines, Carmustines with Polifeprosans, Celecoxibs, Chlorambucils, Cisplatins, Cladribines, Cyclophosphamides, Cytarabines, Cytarabine liposomals, Dacarbazines, Dactinomycins, Daunorubicin liposomals, Daunorubicins, Daunomycins, Denileukin Diftitoxes, Dexrazoxanes, Docetaxels, Doxorubicins, Doxorubicin liposomals, Dromostanolone propionates, Elliott's B Solutions, Epirubicins, Epoetin alfas, Estramustines, Etoposides, Etoposide phosphates, Etoposide VP-16s, Exemestanes, Floxuridines, Fludarabines, Fluorouracils, 5-Fluorouracils, Fulvestrants, Gemcitabines, Gemtuzumabs, Ozogamicins, Gemtuzumab ozogamicins, Hydroxyureas, Idarubicins, Ifosfamides, Imatinib mesylates, Irinotecans, Letrozoles, Leucovorins, Levamisoles, Lomustines, CCNUs, Mechlorethamines, Nitrogen mustards, Megestrols, Megestrol acetates, Melphalans, L-PAMs, Mercaptopurines, 6-Mercaptopurines, Mesnas, Methotrexates, Methoxsalens, Mitomycins, Mitomycin C's, Mitotanes, Mitoxantrones, Nandrolones, Nandrolone Phenpropionates, Nofetumomabs, Oprelvekins, Oxaliplatins, Paclitaxels, Pamidronates, Pegademases, Pentostatins, Pipobromans, Plicamycins, Mithramycins, Porfimers, Porfimer sodiums, Procarbazines, Quinacrines, Rasburicases, Rituximabs, Sargramostims, Streptozocins, Talcs, Tamoxifens, Temozolomides, Teniposides, Testolactones, Thioguanines, 6-Thioguanines, Triethylenethiophosphoramides (Thiotepas), Topotecans, Toremifenes, Trastuzumabs, Tretinoins, Uracil Mustards, Valrubicins, Vinblastines, Vincristines, Vinorelbines, Zoledronates, Acivicins, Aclarubicins, Acodazoles, Acronines, Adozelesins, Aldesleukins, Retinoic Acids, Alitretinoins, 9-Cis-Retinoic Acids, Alvocidibs, Ambazones, Ambomycins, Ametantrones, Aminoglutethimides, Amsacrines, Anaxirones, Ancitabines, Anthramycins, Apaziquones, Argimesnas, Asperlins, Atrimustines, Azacitidines, Azetepas, Azotomycins, Banoxantrones, Batabulins, Batimastats, Benaxibines, Bendamustines, Benzodepas, Bicalutamides, Bietaserpines, Biricodars, Bisantrenes, Bisnafide Dimesylates, Bizelesins, Bortezomibs, Brequinars, Bropirimines, Budotitanes, Cactinomycins, Canertinibs, Caracemides, Carbetimers, Carboquones, Carmofurs, Carubicins, Carzelesins, Cedefingols, Cemadotins, Chlorambucils, Cioteronels, Cirolemycins, Clanfenurs, Clofarabines, Crisnatols, Decitabines, Dexniguldipines, Dexormaplatins, Dezaguanines, Diaziquones, Dibrospidiums, Dienogests, Dinalins, Disermolides, Dofequidars, Doxifluridines, Droloxifenes, Duazomycins, Ecomustines, Edatrexates, Edotecarins, Eflomithines, Elacridars, Elinafides, Elsamitrucins, Emitefurs, Enloplatins, Enpromates, Enzastaurins, Epipropidines, Eptaloprosts, Erbulozoles, Esorubicins, Etanidazoles, Etoglucids, Etoprines, Exisulinds, Fadrozoles, Fazarabines, Fenretinides, Fluoxymesterones, ~~Fluorocitabines~~Flurocitabines, Fosquidones, Fostriecins, Fotretamines, Galarubicins, Galocitabines, Geroquinols, Gimatecans, Gimeracils, Gloxazones, Glufosfamides, Ilmofosines, Ilomastats, Imexons, Improsulfans, Indisulams, Inproquones, Interleukins, Interleukin-2s, recombinant Interleukins, Intoplicines, Iobenguanes, Iproplatins, Irsogladines, Ixabepilones, Ketotrexates, L-Alanosines, Lanreotides, Lapatinibs, Ledoxantrones, Leuprolides, Leuprorelins, Lexacalcitols, Liarozoles, Lobaplatins, Lometrexols, Lonafarnibs, Losoxantrones, Lurtotecans, Mafosfamides, Mannosulfans, Marimastats, Masoprocols, Maytansines, Mechlorethamines, Melengestrols, Melphalans, Menogarils, Mepitiostanes, Metesinds,

Metomidates, Metoprines, Meturedepas, Miboplatins, Miproxifenes, Misonidazoles, Mitindomides, Mitocarcins, Mitocromins, Mitoflaxones, Mitogillins, Mitoguazones, Mitomalcins, Mitonafides, Mitoquidones, Mitospers, Mitozolomides, Mivobulins, Mizoribines, Mofarotenes, Mopidamols, Mubritinibs, Mycophenolic Acids, Nedaplatins, Neizarabines, Nemorubicins, Nitracrines, Nocodazoles, Nogalamycins, Nolatrexeds, Nortopixantrones, Ormaplatins, Ortataxels, Oteracils, Oxisurans, Oxophenarsines, Patupilones, Peldesines, Peliomycins, Pelitrexols, Pemetrexeds, Pentamustines, Peplomycins, Perfosfamides, Perifosines, Picoplatins, Pinafides, Piposulfans, Pirfenidones, Piroxantrones, Pixantrones, Plevitrexeds, Plomestanes, Porfiromycins, Prednimustines, Propamidines, Prospidiums, Pumitepas, Puromycins, Pyrazofurins, Ranimustines, Riboprines, Ritrosulfans, Rogletimides, Roquinimexs, Rufocromomycins, Sabarubicins, Safingols, Satraplatins, Sebriplatins, Semustines, Simtrazenes, Sizofirans, Sobuzoxanes, Sorafenibs, Sparfosates, Sparfosic Acids, Sparsomycins, Spirogermaniums, Spiromustines, Spiroplatins, Squalamines, Streptonigrins, Streptovarycins, Sufosfamides, Sulofenurs, Tacedinalines, Talisomycins, Tallimustines, Tariquidars, Tauromustines, Tecogalans, Tegafurs, Teloxantrones, Temoporfins, Teroxirones, Thiamiprines, Tiamiprines, Tiazofurins, Tilomisoles, Tilorones, Timcodars, Timonacics, Tirapazamines, Topixantrones, Trabectedins, Ecteinascidin 743, Trestolones, Triciribines, Trilostanes, Trimetrexates, Triplatin Tetranitrates, Triptorelins, Trofosfamides, Tubulozoles, Ubenimexs, Uredepas, Valspodars, Vapreotides, Verteporfins, Vinblastines, Vindesines, Vinepidines, Vinflunines, Vinformides, Vinglycinates, Vinleucinols, Vinleurosines, Vinrosidines, Vintriptols, Vinzolidines, Vorozoles, Xanthomycin A's, Guamecyclines, Zeniplatins, Zilascorbs [2-H], Zinostatins, Zorubicins, Zosuquidars, Acetazolamides, Acyclovirs, Adipiodones, Alatrofloxacins, Alfentanils, Allergenic extracts, Alpha 1-proteinase inhibitors, Alprostadils, Amikacins, Amino acids, Aminocaproic acids, Aminophyllines, Amitriptylines, Amobarbitals, Amrinones, Analgesics, Anti-poliomyelitic vaccines, Anti-rabic serums, Anti-tetanus immunoglobulins, tetanus vaccines, Antithrombin IIIs, Antivenom serums, Argatrobans, Arginines, Ascorbic acids, Atenolols, Atracuriums, Atropines, Aurothioglucoses, Azathioprines, Aztreonams, Bacitracins, Baclofens, Basiliximabs, Benzoic acids, Benztropines, Betamethasones, Biotins, Bivalirudins, Botulism antitoxins, Bretyliums, Bumetanides, Bupivacaines, Buprenorphines, Butorphanols, Calcitonins, Calcitriols, Calciums, Capreomycins, Carboprosts, Carnitines, Cefamandoles, Cefoperazones, Cefotaximes, Cefoxitins, Ceftizoximes, Cefuroximes, Chloramphenicols, Chloroprocaines, Chloroquines, Chlorothiazides, Chlorpromazines, Chondroitinsulfuric acids, Choriogonadotropin alfas, Chromiums, Cidofovirs, Cimetidines, Ciprofloxacins, Cisatracuriums, Clonidines, Codeines, Colchicines, Colistins, Collagens, Corticorelin ovine triflutates, Corticotrophins, Cosyntropins, Cyanocobalamins, Cyclosporines, Cysteines, Dacliximabs, Dalfopristins, Dalteparins, Danaparoids, Dantrolenes, Deferoxamines, Desmopressins, Dexamethasones, Dexmedetomidines, Dexpanthenols, Dextrans, Iron dextrans, Diatrizoic acids, Diazepams, Diazoxides, Dicyclomines, Digibinds, Digoxins, Dihydroergotamines, Diltiazems, Diphenhydramines, Dipyridamoles, Dobutamines, Dopamines, Doxacuriums, Doxaprams, Doxercalciferols, Doxycyclines, Droperidols, Dyphyllines, Edetic acids, Edrophoniums, Enalaprilats, Ephedrines, Epoprostenols, Ergocalciferols, Ergonovines, Ertapenems, Erythromycins, Esmolols, Estradiols, Estrogenics, Ethacrynic acids, Ethanolamines, Ethanols, Ethiodized oils, Etidronic acids, Etomidates, Factor VIIIs, Famotidines, Fenoldopams, Fentanyls, Flumazenils, Fluoresceins, Fluphenazines, Folic acids, Fomepizoles, Fomivirsens, Fondaparinuxs, Foscarnets, ~~Fosphenyloins~~Fosphenytoins, Furosemides, Gadoteridols, Gadoversetamides, Ganciclovirs, Gentamicins, Glucagons,

Glucoses, Glycines, Glycopyrrolates, Gonadorelins, Gonadotropin chorionics, Haemophilus B polysaccharides, Hemins, Herbals, Histamines, Hydralazines, Hydrocortisones, Hydromorphones, Hydroxocobalamins, Hydroxyzines, Hyoscyamines, Ibutilides, Imiglucerases, Indigo carmines, Indomethacins, Iodides, Iopromides, Iothalamic acids, Ioxaglic acids, Ioxilans, Isoniazids, Isoproterenols, Japanese encephalitis vaccines, Kanamycins, Ketamines, Labetalols, Lepirudins, Levobupivacaines, Levothyroxines, Lincomycins, Liothyronines, Luteinizing hormones, Lyme disease vaccines, Mangafodipirs, Manthtols, Meningococcal polysaccharide vaccines, Meperidines, Mepivacaines, Mesoridazines, Metaraminols, Methadones, Methocarbamols, Methohexitals, Methyldopates, Methylergonovines, Metoclopramides, Metoprolols, Metronidazoles, Minocyclines, Mivacuriums, Morrhuic acids, Moxifloxacins, Muromonab-CD3s, Mycophenolate mofetils, Nafcillins, Nalbuphines, Nalmefenes, Naloxones, Neostigmines, Niacinamides, Nicardipines, Nitroglycerins, Nitroprussides, Norepinephrines, Orphenadrines, Oxacillins, Oxymorphones, Oxytetracyclines, Oxytocins, Pancuroniums, Panthenols, Pantothenic acids, Papaverines, Peginterferon-alpha (e.g., interferon alpha 2a or 2b), Penicillin Gs, Pentamidines, Pentazocines, Pentobarbitals, Perflutrens, Perphenazines, Phenobarbitals, Phentolamines, Phenylephrines, ~~Phenyloins~~Phenytoins, Physostigmines, Phytonadiones, ~~Polymyxin bs~~Polymyxinbs, Pralidoximes, ~~Prilocalnes~~Prilocaines, Procainamides, Procaines, Prochlorperazines, Progesterones, Propranolols, Pyridostigmine hydroxides, Pyridoxines, Quinidines, Quinupristins, Rabies immunoglobulins, Rabies vaccines, Ranitidines, Remifentanils, Riboflavins, Rifampins, Ropivacaines, Samariums, Scopolamines, Seleniums, Sermorelins, Sincalides, Somatrems, Spectinomycins, Streptokinases, Streptomycins, Succinylcholines, Sufentanils, Sulfamethoxazoles, Tacrolimuses, Terbutalines, Teriparatides, Testosterones, Tetanus antitoxins, Tetracaines, Tetradecyl sulfates, Theophyllines, Thiamines, Thiethylperazines, Thiopentals, Thyroid stimulating hormones, Tinzaparins, Tirofibans, Tobramycins, Tolazolines, Tolbutamides, Torsemides, Tranexamic acids, Treprostinils, Trifluoperazines, Trimethobenzamides, Trimethoprims, Tromethamines, Tuberculins, Typhoid vaccines, Urofollitropins, Urokinases, Valproic acids, Vasopressins, Vecuroniums, Verapamils, Voriconazoles, Warfarins, Yellow fever vaccines, Zidovudines, Zincs, Ziprasidone hydrochlorides, Aclacinomycins, Actinomycins, Adriamycins, Azaserines, 6-Azauridines, Carzinophilins, Chromomycins, Denopterins, 6-Diazo-5-Oxo-L-Norleucines, Enocitabines, Floxuridines, Olivomycins, Pirarubicins, Piritrexims, Pteropterins, Tegafurs, Tubercidins, Alteplases, Arcitumomabs, bevacizumabs, Botulinum Toxin Type A's, Botulinum Toxin Type B's, Capromab Pendetides, Daclizumabs, Dornase alfas, Drotrecogin alfas, Imciromab Pentetates, and Iodine-131's.

~~[0563]~~(459) Delivery of Insulin

~~[0564]~~(460) Methods provided herein include methods of co-administering a modified PH20 polypeptide and an insulin to increase subcutaneous delivery of the insulin, such as a fast-acting insulin (see e.g., U.S. Pat. ~~No~~Nos. 7,767,429; ~~U.S. Pat. No.~~ 7,846,431; U.S. Publication No. US20090304665; and U.S. application Ser. Nos. 13/507,263; 13/507,262 and 13/507,261). Such methods include methods of direct administration, and pump and continuous infusion methods, including open and closed pump systems. For example, exemplary insulins that can be administered with a modified PH20 hyaluronidase provided herein are fast-acting insulins or insulin analogs. For example, a co-administered insulin includes a regular insulin, insulin aspart, insulin lispro, insulin glulisine or other similar analog variants. Exemplary insulins are insulins that contain an A chain set forth in SEQ ID NO:862 and a B chain set forth in SEQ ID NO:863

or variants that contain one or more amino acid modifications compared to a human insulin set forth in SEQ ID NO: 862 and 863 (A and B chains). For example, exemplary insulin analogs are known to one of skill in the art, and include, but are not limited to, those set forth in SEQ ID NOS:862 (A-chain) and having a B-chain set forth in any of SEQ ID NOS: 865-867.

[0565](461) The co-formulations can be administered subcutaneously to treat any condition that is amenable to treatment with insulin. Therapeutic uses include, but are not limited to, treatment for type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, and for glycemic control in critically ill patients. For example, the co-formulations of a fast acting insulin and hyaluronan degrading enzyme can be administered subcutaneously in discrete doses, such as via a syringe or insulin pen, prior to a meal as prandial insulin therapy in subjects with diabetes to achieve glycemic control. The co-formulations also can be administered subcutaneously or intraperitoneally using an insulin pump or in the context of a closed loop system to continuously control blood glucose levels throughout the day and night and/or to control post-prandial glycemic excursions. It is within the skill of a treating physician to identify such diseases or conditions.

[0566](462) For any disease or condition, including all those exemplified above, for which a fast-acting insulin is indicated or has been used and for which other agents and treatments are available, the co-formulations can be used in combination therewith. Depending on the disease or condition to be treated, exemplary combinations include, but are not limited to, combinations with anti-diabetic drugs, including, but not limited to, sulfonylureas, biguanides, meglitinides, thiazolidinediones, alpha-glucosidase inhibitors, peptide analogs, including glucagon-like peptide (GLP) analogs and, gastric inhibitory peptide (GIP) analogs and DPP-4 inhibitors. In another example, the co-formulations of a fast acting insulin and modified PH20 polypeptide described herein can be administered in combination with, prior to, intermittently with, or subsequent to, one or more other insulins, including fast-acting insulin, and basal-acting insulins.

[0567](463) 2. Methods of Hyaluronan-Associated Diseases and Conditions (e.g., Tumors)

[0568](464) In particular, PH20 hyaluronidase can be used to treat hyaluronan-associated diseases or conditions. Typically, hyaluronan-associated diseases and conditions are associated with elevated hyaluronan expression in a tissue, cell, or body fluid (e.g., tumor tissue or tumor-associated tissue, blood, or interstitial space) compared to a control, e.g., another tissue, cell or body fluid. The elevated hyaluronan expression can be elevated compared to a normal tissue, cell or body fluid, for example, a tissue, cell or body fluid that is analogous to the sample being tested, but isolated from a different subject, such as a subject that is normal (i.e., does not have a disease or condition, or does not have the type of disease or condition that the subject being tested has), for example, a subject that does not have a hyaluronan-associated disease or condition. The elevated hyaluronan expression can be elevated compared to an analogous tissue from another subject that has a similar disease or condition, but whose disease is not as severe and/or is not hyaluronan-associated or expresses relatively less hyaluronan and thus is hyaluronan-associated to a lesser degree. For example, the subject being tested can be a subject with a hyaluronan-associated cancer, where the HA amounts in the tissue, cell or fluid are relatively elevated compared to a subject having a less severe cancer, such as an early stage, differentiated or other type of cancer. In another example, the cell, tissue or fluid contains elevated levels of hyaluronan compared to a control sample, such as a fluid, tissue, extract (e.g.,

cellular or nuclear extract), nucleic acid or peptide preparation, cell line, biopsy, standard or other sample, with a known amount or relative amount of HA, such as a sample, for example a tumor cell line, known to express relatively low levels of HA, such as exemplary tumor cell lines described herein that express low levels of HA, for example, the HCT 116 cell line, the HT29 cell line, the NCI H460 cell line, the DU145 cell line, the Capan-1 cell line, and tumors from tumor models generated using such cell lines.

~~[0569]~~(465) Hyaluronan-associated diseases and conditions include those associated with high interstitial fluid pressure, such as disc pressure, proliferative disorders, such as cancer and benign prostatic hyperplasia, and edema. Edema can result from or be manifested in, for example, organ transplant, stroke or brain trauma. Proliferative disorders include, but are not limited to, cancer, smooth muscle cell proliferation, systemic sclerosis, cirrhosis of the liver, adult respiratory distress syndrome, idiopathic cardiomyopathy, lupus erythematosus, retinopathy, e.g., diabetic retinopathy or other retinopathies, cardiac hyperplasia, reproductive system associated disorders, such as benign prostatic hyperplasia (BPH) and ovarian cysts, pulmonary fibrosis, endometriosis, fibromatosis, hamartomas, lymphangiomatosis, sarcoidosis, desmoid tumors. Cancers include solid and lymphatic/blood tumors and metastatic disease, and undifferentiated tumors. The tumors amenable to treatment typically exhibit cellular and/or stromal expression of a hyaluronan, compared to a non-cancerous tissue of the same tissue type or compared to a non-metastatic tumor of the same tumor-type. Cancers include any one or more of ovarian cancer, in situ carcinoma (ISC), squamous cell carcinoma (SCC), prostate cancer, pancreatic cancer, other gastric cancers, non-small cell lung cancer, breast cancer, brain cancer and colon cancer.

~~[0570]~~(466) Modified PH20 polypeptides provided herein, such as PEGylated forms thereof, can be used to treat tumors. Thus, in addition to its indirect anticancer effects, hyaluronidases also have direct anticarcinogenic effects. Hyaluronidase prevents growth of tumors transplanted into mice (De Maeyer et al., 1992, Int. J. Cancer 51:657-660) and inhibits tumor formation upon exposure to carcinogens (Pawlowski et al., 1979, Int. J. Cancer 23:105-109; Haberman et al., 1981, Proceedings of the 17th Annual Meeting of the American Society of Clinical Oncology, Washington, D.C., 22:105, abstract no. 415). PH20 hyaluronidase has been shown to treat various tumors (see e.g., U.S. Publication No. US2010/0003238 and U.S. application Ser. No. 13/135,817, published as U.S. Publication No. US20120020951).

~~[0571]~~(467) The hyaluronan-rich cancer can be a cancer in which the cancer cells produce ~~HALOs~~HALOS, cancers that have elevated expression of hyaluronan (as determined by immunostaining, e.g., histological staining of sections from the tumor), cancers that have elevated HAS2 (Hyaluronan synthase 2), cancers that do not produce hyaluronidase (HYAL1) in vitro. Hyaluronan-rich cancers can be identified by any method for assessing hyaluronan expression, and other known methods for assaying protein/mRNA expression.

~~[0572]~~(468) Several hyaluronan-rich cancers have been identified. In some cases, hyaluronan expression correlates with poor prognosis, for example, decreased survival rate and/or recurrence-free survival rate, metastases, angiogenesis, cancer cell invasion into other tissues/areas, and other indicators of poor prognosis. Such correlation has been observed, for example, in hyaluronan-rich tumors including ovarian cancer, SCC, ISC, prostate cancer, lung cancer, including non-small-cell lung cancer (NSCLC), breast cancer, colon cancer and pancreatic cancer (see, for example, Anttila et al., Cancer Research, 60:150-155 (2000);

Karvinen et al., British Journal of Dermatology, 148:86-94 (2003); Lipponen et al., Eur. Journal of Cancer, 849-856 (2001); Pirinen et al., Int. J. Cancer: 95: 12-17 (2001); Auvinen et al., American Journal of Pathology, 156(2):529-536 (2000); Ropponen et al., Cancer Research, 58: 342-347 (1998)). Thus, hyaluronan-rich cancers can be treated by administration of a hyaluronidase, such as a soluble PH20, to treat one or more symptoms of the cancer. Hyaluronan-rich tumors include, but are not limited to those of the prostate, breast, colon, ovarian, stomach, head and neck and other tumors and cancers.

~~[0573]~~(469) Other hyaluronan-associated diseases or conditions that are associated with excess glycosaminoglycans and that can be treated with a modified PH20 polypeptide provided herein include, but are not limited to, cardiovascular disease (e.g., following ischemia reperfusion; in arteriosclerosis); vitrectomy and ophthalmic disorders and conditions (e.g., in methods to liquefy the vitreous humor of the eye; reduce postoperative pressure; other ocular surgical procedures such as glaucoma, vitreous and retina surgery and in corneal transplantation); in hypodermoclysis (i.e., infusion of fluids and electrolytes into the hypodermis of the skin); cosmetic applications (e.g., in the treatment of cellulite, "“pigskin"” edema or "“orange peel"” edema); organ transplantation (e.g., associated with interstitial edemas in connection with grafting of an organ); pulmonary disease.

~~[0574]~~(470) 3. Other Uses

~~[0575]~~(471) In further examples of its therapeutic use, modified PH20 polypeptides provided herein, can be used for such purposes as an antidote to local necrosis from paravenous injection of necrotic substances such as vinca alkaloids (Few et al. (1987) Amer. J. Matern. Child Nurs. 12, 23-26), treatment of ganglion cysts (Paul et al. (1997) J Hand Surg. 22 (2): 219-21) and treatment of tissue necrosis due to venous insufficiency (Elder et al. (1980) Lancet 648-649). Modified PH20 polypeptides also can be used to treat ganglion cysts (also known as a wrist cyst, Bible cyst, or dorsal tendon cyst), which are the most common soft tissue mass of the hand and are fluid filled sacs that can be felt below the skin.

~~[0576]~~(472) Modified PH20 polypeptides can be used in the treatment of spinal cord injury by degrading chondroitin sulfate proteoglycans (CSPGs). Following spinal cord injury, glial scars containing CSPGs are produced by astrocytes. CSPGs play a crucial role in the inhibition of axon growth. In addition, the expression of CSPG has been shown to increase following injury of the central nervous system (CNS). Soluble PH20 also can be utilized for the treatment of herniated disks in a process known as chemonucleolysis. Chondroitinase ABC, an enzyme cleaving similar substrates as hyaluronidase, can induce the reduction of intradiscal pressure in the lumbar spine. There are three types of disk injuries. A protruded disk is one that is intact but bulging. In an extruded disk, the fibrous wrapper has torn and the NP has oozed out, but is still connected to the disk. In a sequestered disk, a fragment of the NP has broken loose from the disk and is free in the spinal canal. Chemonucleolysis is typically effective on protruded and extruded disks, but not on sequestered disk injuries.

~~[0577]~~(473) 4. Contraception

~~[0578]~~(474) Modified PH20 polypeptides provided herein can be used as vaccines in contraceptive applications. PH20 is present in the male reproductive tract, and is expressed in

both the testis and epididymis and is present in sperm. PH20 plays a role in fertilization by facilitating entry of the sperm through the cumulus layer surrounding the unfertilized egg. PH20 also is able to bind to hyaluronic acid (HA) on the zona pellucida during early phases of fertilization. This binding also initiates intracellular signaling that aids in the acrosome reaction. Immunization with PH20 has been show to be an effective contraceptive in male guinea pigs (Primakoff et al. (1988) Nature 335:543-546, Tung et al. (1997) Biol. Reprod. 56:1133-1141). It also has been shown to be an effective contraceptive in female guinea pigs due to the generation of anti-PH20 antibodies that prevent sperm and egg binding. In examples herein, the modified PH20 polypeptides can be inactive enzymes, such as any described in Sections C.2. The polypeptides can be administered directly or can be administered as a recombinant virus to deliver the antigen.

I. ExamplesEXAMPLES

[0579](475) The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1

[0580] Generation of Recombinant Human PH20 Hyaluronidase (rHuPH20)

(476) A. Generation of a Soluble rHuPH20-Expressing Cell Line

[0581](477) A recombinant human PH20 hyaluronidase designated rHuPH20 was generated as described in published U.S. Publication No. US20110053247. Briefly, the pCI-PH20-IRES-DHFR-SV40pa (HZ24) plasmid (set forth in SEQ ID NO:5) was used to transfect Chinese Hamster Ovary (CHO cells) (see e.g., U.S. Pat. Nos. 7,767,429 and 7,781,607 and U.S. Publication No. 2006-0104968). The HZ24 plasmid vector for expression of soluble rHuPH20 contains a pCI vector backbone (Promega), DNA encoding amino acids 1-482 of human PH20 hyaluronidase (SEQ ID NO:2), an internal ribosomal entry site (IRES) from the ECMV virus (Clontech), and the mouse dihydrofolate reductase (DHFR) gene. The pCI vector backbone also includes DNA encoding the Beta-lactamaseBeta-lactamase resistance gene (AmpR), an f1fl origin of replication, a Cytomegalovirus immediate-early enhancer/promoter region (CMV), a chimeric intron, and an SV40 late polyadenylation signal (SV40). The DNA encoding the soluble rHuPH20 construct contains an NheI site and a Kozak consensus sequence prior to the DNA encoding the methionine at amino acid position 1 of the native 35 amino acid signal sequence of human PH20, and a stop codon following the DNA encoding the tyrosine corresponding to amino acid position 482 of the human PH20 hyaluronidase set forth in SEQ ID NO:2, followed by a BamHI restriction site.

[0582](478) Non-transfected DG44 CHO cells growing in GIBCO Modified CD-CHO media for DHFR(--) cells, supplemented with 4 mM Glutamine and 18 mL/L Plurionic F68/L (Gibco), were seeded at 0.5.times.×10.sup.6 cells/mL in a shaker flask in preparation for transfection. Cells were grown at 37.degree.° C. in 5% CO.sub.2 in a humidified incubator, shaking at 120 rpm. Exponentially growing non-transfected DG44 CHO cells were tested for viability prior to transfection.

[0583](479) Sixty million viable cells of the non-transfected DG44 CHO cell culture were pelleted and resuspended to a density of 2.times.×10.sup.7 cells in 0.7 mL of 2.times.× transfection buffer (2.times.×HeBS: 40 mM Hepes, pH 7.0, 274 mM NaCl, 10 mM KCl, 1.4 mM Na.sub.2HPO.sub.4, 12 mM dextrose). To each aliquot of resuspended cells, 0.09 mL (250 .mu.gμg) of the linear HZ24 plasmid (linearized by overnight digestion with Cla I (New England Biolabs) was added, and the cell/DNA solutions were transferred into 0.4 cm gap BTX (Gentronics) electroporation cuvettes at room temperature. A negative control electroporation was performed with no plasmid DNA mixed with the cells. The cell/plasmid mixes were electroporated with a capacitor discharge of 330 V and 960 .mu.FμF or at 350 V and 960 μF.mu.F.

[0584](480) The cells were removed from the cuvettes after electroporation and transferred into 5 mL of Modified CD-CHO media for DHFR(--) cells, supplemented with 4 mM Glutamine and 18 mL/L Plurionic F68/L (Gibco), and allowed to grow in a well of a 6-well tissue culture plate without selection for 2 days at 37.degree.° C. in 5% CO.sub.2 in a humidified incubator.

[0585](481) Two days post-electroporation, 0.5 mL of tissue culture media was removed from each well and tested for the presence of hyaluronidase activity, using the microturbidity assay described in Example 8. The results are set forth in Table 6.

(482) TABLE-US-00006 TABLE 6 Initial Hyaluronidase Activity of HZ24 Transfected DG44 CHO cells at 40 hours post-transfection Dilution Activity (Units/mL) Transfection 1 330 V 1 to 10 0.25 Transfection 2 350 V 1 to 10 0.52 Negative Control 1 to 10 0.015

[0586](483) Cells from Transfection 2 (350V) were collected from the tissue culture well, counted and diluted to 1.times.×10.sup.4 to 2.times.×10.sup.4 viable cells per mL. A 0.1 mL aliquot of the cell suspension was transferred to each well of five, 96 well round bottom tissue culture plates. One hundred microliters of CD-CHO media (GIBCO) containing 4 mM GlutaMAX.TM.™-1 supplement (GIBCO.TM.™, Invitrogen Corporation) and without hypoxanthine and thymidine supplements were added to the wells containing cells (final volume 0.2 mL). Ten clones were identified from the 5 plates grown without methotrexate (Table 7).

(484) TABLE-US-00007 TABLE 7 Hyaluronidase activity of identified clones Plate/Well ID Relative Hyaluronidase 1C3 261 2C2 261 3D3 261 3E5 243 3C6 174 2G8 103 1B9 304 2D9 273 4D10 302

[0587](485) Six HZ24 clones were expanded in culture and transferred into shaker flasks as single cell suspensions. Clones 3D3, 3E5, 2G8, 2D9, 1E11, and 4D10 were plated into 96-well round bottom tissue culture plates using a two-dimensional infinite dilution strategy in which cells were diluted 1:2 down the plate, and 1:3 across the plate, starting at 5000 cells in the top left hand well. Diluted clones were grown in a background of 500 non-transfected DG44 CHO cells per well, to provide necessary growth factors for the initial days in culture. Ten plates were made per subclone, with 5 plates containing 50 nM methotrexate and 5 plates without methotrexate.

[0588](486) Clone 3D3 produced 24 visual subclones (13 from the no methotrexate treatment, and 11 from the 50 nM methotrexate treatment). Significant hyaluronidase activity was measured

in the supernatants from 8 of the 24 subclones (>≥50 Units/mL), and these 8 subclones were expanded into T-25 tissue culture flasks. Clones isolated from the methotrexate treatment protocol were expanded in the presence of 50 nM methotrexate. Clone 3D35M was further expanded in 500 nM methotrexate giving rise to clones producing hyaluronidase activity in excess of 1,000 Units/mL in shaker flasks (clone 3D35M; or Gen1 3D35M). A master cell bank (MCB) of the 3D35M cells was then prepared.

(487) B. Production Gen2 Cells Containing Soluble humanHuman PH20 (rHuPH20)

[0589](488) The Gen1 3D35M cell line described in Example 1.A was adapted to higher methotrexate levels to produce generation 2 (Gen2) clones. 3D35M cells were seeded from established methotrexate-containing cultures into CD CHO medium containing 4 mM GlutaMAX-1.TM.™ and 1.0 .mu.MμM methotrexate. The cells were adapted to a higher methotrexate level by growing and passaging them 9 times over a period of 46 days in a 37.degree.° C., 7% CO.sub.2 humidified incubator. The amplified population of cells was cloned out by limiting dilution in 96-well tissue culture plates containing medium with 2.0 .mu.MμM methotrexate. After approximately 4 weeks, clones were identified and clone 3E10B was selected for expansion. 3E10B cells were grown in CD CHO medium containing 4 mM GlutaMAX-1.TM.™ and 2.0 .mu.MμM methotrexate for 20 passages. A master cell bank (MCB) of the 3E10B cell line was created and frozen and used for subsequent studies.

[0590](489) Amplification of the cell line continued by culturing 3E10B cells in CD CHO medium containing 4 mM GlutaMAX-1.TM.™ and 4.0 .mu.MμM methotrexate. After the 12.sup.th passage, cells were frozen in vials as a research cell bank (RCB). One vial of the RCB was thawed and cultured in medium containing 8.0 .mu.MμM methotrexate. After 5 days, the methotrexate concentration in the medium was increased to 16.0 .mu.MμM, then 20.0 .mu.MμM 18 days later. Cells from the 8.sup.th passage in medium containing 20.0 .mu.MμM methotrexate were cloned out by limiting dilution in 96-well tissue culture plates containing CD CHO medium containing 4 mM GlutaMAX-1.TM.™ and 20.0 .mu.MμM methotrexate. Clones were identified 5-6 weeks later and clone 2B2 was selected for expansion in medium containing 20.0 .mu.MμM methotrexate. After the 11.sup.th passage, 2B2 cells were frozen in vials as a research cell bank (RCB).

[0591](490) The resultant 2B2 cells are dihydrofolate reductase deficient (dhfr--) DG44 CHO cells that express soluble recombinant human PH20 (rHuPH20). The soluble PH20 is present in 2B2 cells at a copy number of approximately 206 copies/cell. Southern blot analysis of Spe I-, Xba I- and BamH I/Hind III-digested genomic 2B2 cell DNA using a rHuPH20-specific probe revealed the following restriction digest profile: one major hybridizing band of ~7.7 kb and four minor hybridizing bands (.about.~13.9, .about.~6.6, .about.~5.7 and .about.~4.6 kb) with DNA digested with Spe I; one major hybridizing band of .about.~5.0 kb and two minor hybridizing bands (.about.~13.9 and .about.~6.5 kb) with DNA digested with Xba I; and one single hybridizing band of .about.~1.4 kb observed using 2B2 DNA digested with BamH I/Hind III.

(491) C. Production of Gen2Gent Soluble rHuPH20 in 300 L Bioreactor Cell Culture

[0592](492) A vial of HZ24-2B2 was thawed and expanded from shaker flasks through 36 L spinner flasks in CD-CHO media (Invitrogen, Carlsbad, Calif.) supplemented with 20 .mu.MμM

methotrexate and GlutaMAX-1.TM.™ (Invitrogen). Briefly, the vial of cells was thawed in a 37.degree.° C. water bath, medium was added and the cells were centrifuged. The cells were re-suspendedresuspended in a 125 mL shake flask with 20 mL of fresh medium and placed in a 37.degree.° C., 7% CO.sub.2 incubator. The cells were expanded up to 40 mL in the 125 mL shake flask. When the cell density reached greater than 1.5.times.1 5×10.sup.6 cells/mL, the culture was expanded into a 125 mL spinner flask in a 100 mL culture volume. The flask was incubated at 37.degree.° C., 7% CO.sub.2. When the cell density reached greater than 1.5.times.×10.sup.6 cells/mL, the culture was expanded into a 250 mL spinner flask in 200 mL culture volume, and the flask was incubated at 37.degree.° C., 7% CO.sub.2. When the cell density reached greater than 1.5.times.1 5×10.sup.6 cells/mL, the culture was expanded into a 1 L spinner flask in 800 mL culture volume and incubated at 37.degree.° C., 7% CO.sub.2. When the cell density reached greater than 1.5.times.1 5×10.sup.6 cells/mL the culture was expanded into a 6 L spinner flask in 5000 mL culture volume and incubated at 37.degree.° C., 7% CO.sub.2. When the cell density reached greater than 1.5.times.×10.sup.6 cells/mL the culture was expanded into a 36 L spinner flask in 32 L culture volume and incubated at 37.degree.° C., 7% CO.sub.2.

[0593](493) A 400 L reactor was sterilized and 230 mL of CD-CHO media were added. Before use, the reactor was checked for contamination. Approximately 30 L cells were transferred from the 36 L spinner flasks to the 400 L bioreactor (Braun) at an inoculation density of 4.0.times.×10.sup.5 viable cells per mL and a total volume of 260 L. Parameters were: temperature setpoint, 37.degree.° C.; Impeller Speed 40-55 RPM; Vessel Pressure: 3 psi; Air Sparge 0.5-1.5 L/Min.; Air Overlay: 3 L/min. The reactor was sampled daily for cell counts, pH verification, media analysis, protein production and retention. Also, during the run nutrient feeds were added. At 120 hrs (day 5), 10.4 L of Feed #1 Medium (4.times.×CD-CHO+33 g/L Glucose+160 mL/L Glutamax-1.TM.™+83 mL/L Yeastolate+33 mg/L rHuInsulin) was added. At 168 hours (day 7), 10.8 L of Feed #2 (2.times.×CD-CHO+33 g/L Glucose+80 mL/L Glutamax-1.TM.™+167 mL/L Yeastolate+0.92 g/L Sodium Butyrate) was added, and culture temperature was changed to 36.5.degree.° C. At 216 hours (day 9), 10.8 L of Feed #3 (1.times.lx CD-CHO+50 g/L Glucose+50 mL/L Glutamax-1.TM.™+250 mL/L Yeastolate+1.80 g/L Sodium Butyrate) was added, and culture temperature was changed to 36.degree.° C. At 264 hours (day 11), 10.8 L of Feed #4 (1.times.×CD-CHO+33 g/L Glucose+33 mL/L Glutamax-1.TM.™+250 mL/L Yeastolate+0.92 g/L Sodium Butyrate) was added, and culture temperature was changed to 35.5.degree.° C. The addition of the feed media was observed to dramatically enhance the production of soluble rHuPH20 in the final stages of production. The reactor was harvested at 14 or 15 days or when the viability of the cells dropped below 40%. The process resulted in a final productivity of 17,000 Units per mL with a maximal cell density of 12 million cells/mL. At harvest, the culture was sampled for mycoplasma, bioburden, endotoxin and virus in vitro and in vivo, by Transmission Electron Microscopy (TEM) and enzyme activity.

[0594](494) The culture was pumped by a peristaltic pump through four Millistak filtration system modules (Millipore) in parallel, each containing a layer of diatomaceous earth graded to 4-8 .mu.mµm and a layer of diatomaceous earth graded to 1.4-1.1 .mu.mµm, followed by a cellulose membrane, then through a second single Millistak filtration system (Millipore) containing a layer of diatomaceous earth graded to 0.4-0.11 .mu.mµm and a layer of diatomaceous earth graded to <≤0.1 .mu.mµm, followed by a cellulose membrane, and then through a 0.22 .mu.mµm final filter into a sterile single use flexible bag with a 350 L capacity.

The harvested cell culture fluid was supplemented with 10 mM EDTA and 10 mM Tris to a pH of 7.5. The culture was concentrated 10~~.times.~~× with a tangential flow filtration (TFF) apparatus using four Sartoslice TFF 30 kDa molecular weight cut-off (MWCO) polyether sulfone (PES) filter (Sartorious), followed by a 10~~.times.~~× buffer exchange with 10 mM Tris, 20 mM Na.sub.2SO.sub.4, pH 7.5 into a 0.22 ~~.mu.m~~µm final filter into a 50 L sterile storage bag.

~~[0595]~~(495) The concentrated, diafiltered harvest was inactivated for virus. Prior to viral inactivation, a solution of 10% Triton~~.RTM.~~® X-100, 3% tri (n-butyl) phosphate (TNBP) was prepared. The concentrated, diafiltered harvest was exposed to 1% Triton~~.RTM.~~® X-100, 0.3% TNBP for 1 hour in a 36 L glass reaction vessel immediately prior to purification on the Q column.

(496) D. Purification of ~~Gen2~~Gent Soluble rHuPH20

~~[0596]~~(497) A Q Sepharose (Pharmacia) ion exchange column (9 L resin, H=29 cm, D=20 cm) was prepared. Wash samples were collected for a determination of pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column volumes of 10 mM Tris, 20 mM Na.sub.2SO.sub.4, pH 7.5. Following viral inactivation, the concentrated, diafiltered harvest was loaded onto the Q column at a flow rate of 100 cm/hr. The column was washed with 5 column volumes of 10 mM Tris, 20 mM Na.sub.2SO.sub.4, pH 7.5 and 10 mM Hepes, 50 mM NaCl, pH7.0. The protein was eluted with 10 mM Hepes, 400 mM NaCl, pH 7.0 into a 0.22 ~~.mu.m~~µm final filter into sterile bag. The eluate sample was tested for bioburden, protein concentration and hyaluronidase activity. A.sub.280 absorbance readings were taken at the beginning and end of the exchange.

~~[0597]~~(498) Phenyl-Sepharose (Pharmacia) hydrophobic interaction chromatography was next performed. A Phenyl-Sepharose (PS) column (19-21 L resin, H=29 cm, D=30 cm) was prepared. The wash was collected and sampled for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate, 0.1 mM CaCl.sub.2, pH 7.0. The protein eluate from the Q sepharose column was supplemented with 2M ammonium sulfate, 1 M potassium phosphate and 1 M CaCl.sub.2 stock solutions to yield final concentrations of 5 mM, 0.5 M and 0.1 mM, respectively. The protein was loaded onto the PS column at a flow rate of 100 cm/hr and the column flow thru collected. The column was washed with 5 mM potassium phosphate, 0.5 M ammonium sulfate and 0.1 mM CaCl.sub.2 pH 7.0 at 100 cm/hr and the wash was added to the collected flow thru. Combined with the column wash, the flow through was passed through a 0.22 ~~.mu.m~~µm final filter into a sterile bag. The flow through was sampled for bioburden, protein concentration and enzyme activity.

~~[0598]~~(499) An aminophenyl boronate column (Prometics) was prepared. The wash was collected and sampled for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The PS flow through containing purified protein was loaded onto the aminophenyl boronate column at a flow rate of 100 cm/hr. The column was washed with 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0. The column was washed with 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0. The column was washed with 20 mM bicine, 100 mM sodium chloride, pH 9.0. The protein was eluted with 50 mM Hepes, 100 mM NaCl, pH 6.9 and passed

through a sterile filter into a sterile bag. The eluted sample was tested for bioburden, protein concentration and enzyme activity.

[0599](500) The hydroxyapatite (HAP) column (Biorad) was prepared. The wash was collected and tested for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 mM potassium phosphate, 100 mM NaCl, 0.1 mM CaCl.sub.2, pH 7.0. The aminophenyl boronate purified protein was supplemented to final concentrations of 5 mM potassium phosphate and 0.1 mM CaCl.sub.2 and loaded onto the HAP column at a flow rate of 100 cm/hr. The column was washed with 5 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM CaCl.sub.2. The column was next washed with 10 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM CaCl.sub.2. The protein was eluted with 70 mM potassium phosphate, pH 7.0 and passed through a 0.22 .mu.mµm sterile filter into a sterile bag. The eluted sample was tested for bioburden, protein concentration and enzyme activity.

[0600](501) The HAP purified protein was then passed through a virus removal filter. The sterilized Viosart filter (Sartorius) was first prepared by washing with 2 L of 70 mM potassium phosphate, pH 7.0. Before use, the filtered buffer was sampled for pH and conductivity. The HAP purified protein was pumped via a peristaltic pump through the 20 nM virus removal filter. The filtered protein in 70 mM potassium phosphate, pH 7.0 was passed through a 0.22 .mu.mµm final filter into a sterile bag. The filtered sample was tested for protein concentration, enzyme activity, oligosaccharide, monosaccharide and sialic acid profiling. The sample also was tested for process related impurities.

[0601](502) The protein in the filtrate was then concentrated to 10 mg/mL using a 10 kDa molecular weight cut off (MWCO) Sartocon Slice tangential flow filtration (TFF) system (Sartorius). The filter was first prepared by washing with 10 mM histidine, 130 mM NaCl, pH 6.0 and the permeate was sampled for pH and conductivity. Following concentration, the concentrated protein was sampled and tested for protein concentration and enzyme activity. A 6.times.× buffer exchange was performed on the concentrated protein into the final buffer: 10 mM histidine, 130 mM NaCl, pH 6.0. Following buffer exchange, the concentrated protein was passed though a 0.22 .mu.mµm filter into a 20 L sterile storage bag. The protein was sampled and tested for protein concentration, enzyme activity, free sulfydrylsulfhydryl groups, oligosaccharide profiling and osmolality. Lot number WRS2 was used as a standard in the assays described below, the results showed that the test description for appearance was clear and colorless; the pH was 7.4; the endotoxin level was <≤0.01 EU/mL; the osmolality was 308 mOsm/Kg; the density was 1.005 g/mL; the rHuPH20 content was 1.3 ppm; and the hyaluronidase activity was 145 USP U/mL.

[0602](503) The sterile filtered bulk protein was then asceptically aseptically dispensed at 20 mL into 30 mL sterile Teflon vials (Nalgene). The vials were then flash frozen and stored at -20.+-.−20±5.degree.° C.

Example 2

Generation of PH20 Mutant Library

[0603](504) A. Cloning and Mutagenesis

[0604](505) In this example, a human hyaluronidase PH20 library was created by cloning DNA encoding human PH20 into a plasmid followed by transfection and protein expression.

[0605](506) The library was created by mutagenesis of a PH20 template that is a codon optimized version of PH20 with an Ig Kappa leader sequence. Specifically, for generating the library of variants, the HZ24-PH20(OHO)-IRES-SEAP expression vector (set forth in SEQ ID NO:4) was used as a template, which contains the sequence of nucleotides encoding PH20 set forth in SEQ ID NO:1, which encodes a precursor PH20 set forth in SEQ ID NO:2 or a mature PH20 set forth in SEQ ID NO:3 lacking residues 1-22 corresponding to the IgK signal sequence. The backbone of the vector was derived from the original HZ24 vector containing the DHFR selection marker (see Example 1 and SEQ ID NO:5) with the addition of an IgK leader sequence and codon optimization. The expression vector also was modified to contain the gene for secreted alkaline phosphatase (SEAP). Hence, in addition to sequence encoding PH20, the HZ24-PH20(OHO)-IRES-SEAP expression vector also contains an internal ribosome entry site (EMCV IRES) that is linked to the coding sequence for the gene for secreted alkaline phosphatase (SEAP), and a single CMV promoter that drives expression of PH20 and SEAP in the construct. It also contains a gene for ampilcillinampicillin resistance. With reference to the sequence of nucleotides set forth in SEQ ID NO:4, the sequence of nucleotides encoding PH20 corresponds to nucleotides 1058-2464 (including the IgK leader sequence), the sequence of nucleotides encoding SEAP corresponds to nucleotides 2970-4529, and the ampicillin resistance gene corresponds to nucleotides 5778-6635.

[0606](507) The first library was made to generate encoded variant proteins wherein each of residues 23-469 of SEQ ID NO:2 (corresponding to residues 1-447 of SEQ ID NO:3 or residues 36-482 of SEQ ID NO:6) was changed to one of about 15 amino acid residues, such that each member contained a single amino change. The resulting library contained 6753 variant members, each containing a single amino acid mutation compared to residues 23-469 of SEQ ID NO:2 (corresponding to residues 1-447 of SEQ ID NO:3 or residues 36-482 of SEQ ID NO:6). Glycerol stocks of the resulting library were prepared and stored at -80.degree.−80° C. The amino acid replacements (mut) in each member are listed in Table 8 below, and correspond to amino acid replacements with reference to the sequence of amino acids of PH20 set forth in SEQ ID NO:3 (and SEQ ID NOS: 7 or 32-66, which are the mature sequence of PH20 or other C-terminally truncated fragments thereof). The corresponding mutated codons (cod) of each PH20 variant in the library are also listed in Table 8, and correspond to nucleotide residue changes in the corresponding encoding nucleotide for PH20 set forth as 1058-2464 of SEQ ID NO:4. Each member was expressed and screened for hyaluoridasehyaluorindase activity as described below.

(508) TABLE-US-00008 TABLE 8 PH20 Variants mut cod mut cod mut cod mut cod mut cod mut cod L001A GCG Y066S AGT R132N AAT G198T ACT V265G GGT I331K AAG L001C TGT Y066T ACG R132P CCT G198V GTT V265H CAT I331L CTG L001D GAT Y066V GTG R132Q CAG G198W TGG V265I ATT I331Q CAG L001E GAG I067C TGT R132S AGT G198Y TAT V265K AAG I331R CGT L001F TTT I067D GAT R132T ACT Y199A GCG V265L CTG I331S AGT L001G GGT I067E GAG R132V GTG Y199C TGT V265M ATG I331T ACT L001H CAT I067F TTT R132Y TAT Y199E GAG V265N AAT I331W TGG L001K AAG I067G GGG S133A GCT Y199G GGG V265P CCT I331Y TAT L001N AAT I067H CAT S133D GAT Y199H CAT V265Q CAG I332A GCT L001P CCG I067L TTG S133E GAG Y199I ATT V265R AGG I332C TGT L001Q CAG I067N AAT S133F TTT

Y199K AAG V265S TCT I332D GAT L001R CGG I067P CCG S133G GGG Y199L CTT V265W TGG I332E GAG L001S TCT I067Q CAG S133H CAT Y199N AAT V265Y TAT I332F TTT L001T ACG I067R CGG S133I ATT Y199P CCT F266A GCG I332G GGT L001V GTG I067T ACG S133L CTG Y199Q CAG F266C TGT I332H CAT L001W TGG I067V GTT S133M ATG Y199R AGG F266D GAT I332K AAG N002A GCT I067W TGG S133N AAT Y199S TCG F266G GGG I332L CTG N002C TGT I067Y TAT S133P CCT Y199T ACG F266H CAT I332N AAT N002F TTT D068A GCT S133R CGG Y199W TGG F266L CTT I332P CCT N002G GGG D068C TGT S133T ACT N200A GCT F266M CCG I332R AGG N002H CAT D068E GAG S133V GTT N200D GAT F266P ATG I332S AGT N002I ATT D068G GGG S133W TGG N200F CAG F266Q CAG I332T ACT N002K AAG D068H CAC I134A GCT N200G GGT F266R CGG I332Y TAT N002L TTG D068I ATT I134C TGT N200H CAT F266S TCG N333A GCT N002P CCG D068K AAG I134D GAT N200K AAG F266T ACG N333E GAG N002Q CAG D068L TTG I134F TTT N200L CTG F266V GTG N333G GGT N002S AGT D068P CCT I134G GGG N200M ATG F266W TGG N333H CAT N002T ACG D068Q CAG I134H CAT N200P CCT F266Y TAT N333I ATT N002V GTT D068R CGG I134K AAG N200Q CAG A267D GAT N333K AAG N002W TGG D068S TCG I134L TTG N200R AGG A267E GAG N333L CTG N002Y TAT D068T ACT I134P CCT N200S TCT A267G GGT N333M ATG F003A GCT D068V GTG I134Q CAG N200T ACT A267H CAT N333P CCT F003E GAG D068Y TAT I134R CGT N200V GTG A267I ATT N333R CGG F003G GGG S069A GCT I134S TCG N200W TGG A267K AAG N333S AGT F003H CAT S069C TGT I134T ACT N200Y TAT A267L CTT N333T ACT F003I ATT S069E GAG I134V GTG G201A GCG A267M ATG N333V GTT F003K AAG S069F TTT I134W TGG G201E GAG A267N AAT N333W TGG F003L TTG S069G GGG E135A GCT G201F TTT A267P CCG N333Y TAT F003M ATG S069I ATT E135C TGT G201H CAT A267R AGG V334A GCT F003N AAT S069L CTT E135D GAT G201K AAG A267S TCT V334C TGT F003P CCT S069M ATG E135F TTT G201L CTT A267T GTG V334D GAT F003R CGT S069N AAT E135G GGG G201M ATG A267V ACT V334E GAG F003S TCG S069P CCT E135H CAT G201N AAT A267W TGG V334G GGG F003T ACT S069R CGT E135K AAG G201P CCT Y268A GCT V334H CAT F003V GTG S069T ACG E135L TTG G201Q CAG Y268C TGT V334L TTG F003Y TAT S069V GTT E135N AAT G201R CGT Y268F TTT V334M ATG R004A GCG S069W TGG E135P CCT G201S TCG Y268G GGG V334N AAT R004D GAT S069Y TAT E135Q CAG G201T ACG Y268H CAT V334P CCT R004E GAG I070A GCT E135R CGG G201V GTG Y268K AAG V334Q CAG R004F TTT I070C TGT E135S TCT G201W TGG Y268L CTT V334R AGG R004G GGG I070F TTT E135W TGG S202A GCG Y268N AAT V334S TCT R004I ATT I070G GGG E135Y TAT S202E GAG Y268P CCT V334T ACT R004L TTG I070H CAT L136A GCT S202F TTT Y268Q CAG V334Y TAT R004M ATG I070K AAG L136C TGT S202G GGT Y268R CGT T335A GCT R004N AAT I070L TTG L136D GAT S202H CAT Y268S TCG T335C TGT R004P CCT I070N AAT L136F TTT S202K AAG Y268T ACT T335F TTT R004S TCT I070P CCG L136G GGT S202M ATG Y268V GTG T335G GGT R004T ACG I070Q CAG L136H CAT S202N AAT Y268W TGG T335H CAT R004V GTG I070R CGT L136I ATT S202P CCT T269A GCT T335I ATT R004W TGG I070S TCT L136M ATG S202Q CAG T269C TGT T335K AAG R004Y TAT I070T ACT L136N AAT S202R CGT T269D GAT T335L TTG A005D GAT I070V GTT L136P CCT S202T ACG T269E GAG T335N AAT A005G GGG I070Y TAT L136Q CAG S202V GTT T269G GGT T335P CCT A005H CAT T071A GCT L136R CGT S202W TGG T269K AAG T335Q CAG A005I ATT T071C TGT L136S TCG S202Y TAT

T269L CTG T335S TCT A005L CTT T071D GAT L136T ACT C203A GCG T269M ATG T335V GTG A005M ATG T071E GAG L136W TGG C203D GAT T269N AAT T335W TGG A005N AAT T071G GGG V137A GCT C203E GAG T269P CCG T335Y TAT A005P CCG T071H CAT V137C TGT C203G GGG T269Q CAG L336A GCT A005Q CAG T071L TTG V137E GAG C203H CAT T269R AGG L336E GAG A005R AGG T071M ATG V137F TTT C203L CTT T269S TCG L336F TTT A005S TCG T071N AAT V137G GGG C203M ATG T269V GTG L336G GGG A005T ACG T071P CCT V137H CAT C203N AAT T269Y TAT L336H CAT A005V GTG T071Q CAG V137I ATT C203P CCG R270A GCT L336K AAG A005W TGG T071R CGG V137L TTG C203Q CAG R270C TGT L336M ATG A005Y TAT T071S TCG V137N AAT C203R AGG R270D GAT L336N AAT P006A GCG T071V GTG V137P CCT C203S AGT R270E GAG L336P CCT P006D GAT T071Y TAT V137Q CAG C203T ACT R270F TTT L336R AGG P006E GAG G072A GCT V137R CGT C203V GTG R270G GGG L336S TCT P006F TTT G072C TGT V137S TCT C203W TGG R270H CAT L336T ACT P006G GGG G072D GAT V137T ACT F204A GCG R270I ATT L336V GTG P006H CAT G072E GAG V137W TGG F204C TGT R270M ATG L336W TGG P006K AAG G072F TTT V137Y TAT F204E GAG R270N AAT L336Y TAT P006L CTT G072H CAT Q138A GCT F204G GGG R270P CCT A337C TGT P006N AAT G072I ATT Q138C TGT F204H CAT R270Q CAG A337F TTT P006Q CAG G072K AAG Q138E GAG F204I ATT R270S TCG A337G GGG P006R AGG G072L TTG Q138F TTT F204K AAG R270T ACT A337H CAT P006S AGT G072M ATG Q138G GGG F204L CTT R270V GTG A337I ATT P006T ACG G072P CCT Q138H CAT F204M ATG R270Y TAT A337K AAG P006V GTG G072Q CAG Q138I ATT F204P CCT I271A GCT A337L TTG P006W TGG G072R CGG Q138L TTG F204Q CAG I271D GAT A337M ATG P006Y TAT G072S TCT Q138M ATG F204R AGG I271E GAG A337N AAT P007A GCT G072T ACT Q138N AAT F204S AGT I271F TTT A337P CCT P007C TGT G072V GTG Q138R CGT F204T ACT I271G GGG A337R CGG P007D GAT G072W TGG Q138S AGT F204V GTG I271H CAT A337S TCT P007F TTT G072Y TAT Q138V GTT F204W TGG I271K AAG A337T ACT P007G GGT V073A GCG Q138W TGG N205A GCG I271L CTT A337V GTT P007H CAT V073C TGT Q138Y TAT N205D GAT I271M ATG A337W TGG P007I ATT V073D GAT Q139A GCT N205E GAG I271P CCT A338C TGT P007K AAG V073F TTT Q139C TGT N205F TTT I271R AGG A338D GAT P007L TTG V073G GGG Q139D GAT N205G GGG I271S AGT A338E GAG P007M ATG V073H CAT Q139E GAG N205K AAG I271T ACT A338F TTT P007Q CAG V073K AAG Q139F TTT N205L CTG I271V GTT A338G GGG P007R CGG V073L CTT Q139G GGG N205M ATG I271W TGG A338H CAT P007S AGT V073M ATG Q139H CAT N205P CCT V272A GCT A338I ATT P007T ACT V073P CCG Q139K AAG N205R AGG V272C TGT A338K AAG P007V GTG V073Q CAG Q139L CTG N205S TCG V272D GAT A338L CTT P007W TGG V073R TGG Q139M ATG N205T ACG V272E GAG A338P CCT P007Y TAT V073S TCG Q139P CCT N205V GTG V272G GGG A338Q CAG V008A GCT V073T ACG Q139R CGT N205W TGG V272H CAT A338R CGT V008D GAT V073W CGG Q139S TCT N205Y TAT V272K AAG A338S TCG V008E GAG T074A GCT Q139T ACT V206C TGT V272L TTG A338T ACT V008G GGT T074C TGT Q139V GTG V206D GAT V272M ATG A338V GTG V008H CAT T074E GAG Q140A GCT V206F TTT V272N AAT K339D GAT V008I ATT T074F TTT Q140C TGT V206G GGG V272P CCT K339E GAG V008L TTG T074G GGT Q140D GAT V206H CAT V272R AGG K339F TTT V008M ATG T074H CAT Q140F TTT V206I ATT V272S TCG K339G GGG V008N AAT T074K AAG Q140G GGG V206K AAG V272T ACT K339H CAT V008P CCT T074L TTG

Q140H CAT V206L CTT V272W TGG K339L CTG V008Q CAG T074M ATG Q140I ATT V206M ATG F273A GCT K339M ATG V008R CGG T074N AAT Q140K AAG V206P CCG F273C TGT K339N AAT V008S TCT T074P CCG Q140L TTG V206Q CAG F273D GAT K339P CCT V008T ACT T074R CGG Q140M ATG V206R CGG F273G GGG K339R CGG V008W TGG T074S TCG Q140R CGG V206S TCT F273H CAT K339S AGT I009A GCT T074V GTG Q140S AGT V206T ACG F273I ATT K339T ACT I009C TGT T074W TGG Q140V GTG V206Y TAT F273L CTG K339V GTT I009D GAT V075A GCG Q140W TGG E207A GCT F273P CCT K339W TGG I009E GAG V075C TGT Q140Y TAT E207F TTT F273Q CAG K339Y TAT I009G GGG V075D GAT N141A GCT E207G GGG F273R CGG M340A GCT I009H CAT V075F TTT N141D GAT E207H CAT F273S TCG M340C TGT I009K AAG V075G GGG N141E GAG E207I ATT F273T ACG M340D GAT I009L CTT V075H CAT N141F TTT E207K AAG F273V GTT M340E GAG I009N AAT V075L CTT N141G GGT E207L TTG F273W TGG M340F TTT I009P CCT V075M ATG N141H CAT E207M ATG F273Y TAT M340G GGG I009Q CAG V075N AAT N141L TTG E207P CCG T274A GCG M340H CAT I009R CGG V075P CCG N141M ATG E207Q CAG T274C TGT M340K AAG I009S AGT V075Q CAG N141P CCT E207R AGG T274E GAG M340L CTG I009T ACG V075R CGT N141Q CAG E207S TCT T274F ATG M340P CCT I009V GTT V075S TCT N141R CGT E207T ACG T274G GGG M340R CGG P010D GAT V075T ACT N141S TCT E207V GTT T274H CAT M340S TCG P010E GAG V075W TGG N141T ACT E207W TGG T274L CTG M340T ACT P010F TTT V075Y TAT N141V GTT I208A GCT T274N AAT M340V GTG P010G GGT N076A GCT N141W TGG I208C TGT T274P CCT M340W TGG P010H CAT N076C TGT N141Y TAT I208D GAT T274Q CAG C341A GCT P010I ATT N076D GAT V142C TGT I208E GAG T274R CGT C341E GAG P010L CTT N076F TTT V142D GAT I208G GGG T274S AGT C341G GGG P010M ATG N076G GGG V142E GAG I208K AAG T274V GTT C341H CAT P010N AAT N076I ATT V142G GGG I208L TTG T274W TGG C341K AAG P010Q CAG N076K AAG V142H CAT I208M ATG T274Y TAT C341L TTG P010R CGG N076L CTG V142I ATT I208P CCG D275A GCT C341M ATG P010S TCG N076P CCT V142K AAG I208Q CAG D275C TGT C341N AAT P010T ACT N076Q CAG V142L TTG I208R CGT D275E GAG C341Q CAG P010W TGG N076R CGT V142M ATG I208S AGT D275F TTT C341R AGG P010Y TAT N076S AGT V142N AAT I208T ACG D275G GGG C341S TCT N011A GCG N076T ACT V142P CCT I208V GTG D275I ATT C341T ACT N011C TGT N076V GTT V142Q CAG I208W TGG D275K AAG C341V GTT N011D GAT N076W TGG V142R CGG K209A GCG D275L CTT C341W TGG N011E GAG G077D GAT V142S AGT K209C TGT D275M ATG C341Y TAT N011F TTT G077E GAG V142T ACT K209D GAT D275Q CAG S342A GCT N011G GGG G077F TTT Q143C TGT K209E GAG D275R CGT S342D GAT N011H CAT G077H CAT Q143E GAG K209F TTT D275S TCG S342E GAG N011I ATT G077K AAG Q143F TTT K209G GGT D275T ACT S342F TTT N011K AAG G077L TTG Q143G GGG K209L CTG D275V GTG S342G GGG N011L CTG G077M ATG Q143H CAT K209N AAT D275W TGG S342H CAT N011P CCG G077N AAT Q143I ATT K209P CCG Q276C TGT S342I ATT N011S TCG G077P CCG Q143K AAG K209R CGG Q276D GAT S342K AAG N011T ACG G077Q CAG Q143L TTG K209S AGT Q276E GAG S342L TTG N011W TGG G077R CGT Q143M ATG K209T ACT Q276F TTT S342M ATG N011Y TAT G077S TCG Q143N AAT K209V GTT Q276G GGG S342P CCT V012A GCT G077T ACG Q143P CCT K209W TGG Q276H CAT S342Q CAG V012D GAT G077V GTG Q143R CGG K209Y TAT Q276I ATT S342R CGG V012E GAG G077Y TAT Q143S TCG R210A GCG Q276L CTT S342T ACT

V012G GGG G078A GCG Q143T ACT R210C TGT Q276M ATG S342Y TAT V012H CAT G078C TGT Q143V GTG R210D GAT Q276P CCT Q343C TGT V012I ATT G078D GAT Q143Y TAT R210E GAG Q276R CGT Q343D GAT V012K AAG G078H CAT L144A GCT R210G GGT Q276S AGT Q343E GAG V012L CTT G078I ATT L144E GAG R210K AAG Q276V GTT Q343F TTT V012M ATG G078K AAG L144F TTT R210L CTG Q276W TGG Q343G GGG V012N AAT G078L TTG L144G GGG R210M ATG Q276Y TAT Q343I ATT V012P CCG G078M ATG L144I ATT R210N AAT V277A GCT Q343L CTT V012R AGG G078P CCG L144K AAG R210P CCT V277C TGT Q343M ATG V012S TCG G078Q CAG L144N AAT R210S TCG V277D GAT Q343P CCT V012T ACT G078R AGG L144P CCT R210T ACT V277E GAG Q343R AGG V012W TGG G078S TCG L144Q CAG R210V GTG V277G GGG Q343S AGT P013A GCT G078T ACT L144R CGT R210W TGG V277H CAT Q343T ACT P013E GAG G078V GTG L144S TCT R210Y TAT V277K AAG Q343V GTG P013F TTT G078Y TAT L144T ACT N211A GCG V277L TTG Q343W TGG P013G GGG I079A GCT L144V GTT N211C TGT V277M ATG Q343Y TAT P013H CAT I079D GAT L144W TGG N211F TTT V277N AAT V344E GAG P013I ATT I079F TTT L144Y TAT N211G GGG V277Q CAG V344F TTT P013L CTT I079G GGG S145A GCT N211H CAT V277R AGG V344G GGG P013M ATG I079H CAT S145C TGT N211I ATT V277S TCT V344H CAT P013Q CAG I079K AAG S145D GAT N211K AAG V277T ACT V344I ATT P013R CGT I079L TTG S145E GAG N211L CTG V277Y TAT V344L CTG P013S TCG I079N AAT S145F TTT N211M ATG L278A GCT V344M ATG P013T ACT I079P CCG S145G GGG N211P CCT L278E GAG V344N AAT P013V GTG I079R CGT S145H CAT N211R CGG L278F TTT V344P CCT P013W TGG I079S AGT S145L TTG N211S AGT L278G GGG V344Q CAG P013Y TAT I079T ACT S145M ATG N211T ACT L278H CAT V344R CGT F014A GCG I079V GTT S145N AAT N211V GTT L278I ATT V344S TCG F014D GAT I079W TGG S145P CCT N211W TGG L278K AAG V344T ACT F014E GAG I079Y TAT S145R CGT D212A GCT L278M TTT V344W TGG F014G GGT P080A GCG S145T ACT D212E GAG L278N AAT V344Y TAT F014H CAT P080D GAT S145V GTT D212G GGG L278P CCG L345A GCT F014I ATT P080E GAG S145W TGG D212H CAT L278R CGT L345C TGT F014K AAG P080F TTT L146A GCT D212I ATT L278S TCT L345D GAT F014M ATG P080G GGG L146C TGT D212K AAG L278T ACT L345E GAG F014N AAT P080I ATT L146E GAG D212L CTG L278V GTT L345G GGG F014P CCT P080K AAG L146G GGG D212M ATG L278Y TAT L345H CAT F014Q CAG P080L CTT L146H CAT D212N AAT K279A GCG L345K AAG F014R CGG P080M ATG L146I ATT D212P CCT K279C TGT L345N AAT F014T ACT P080N AAT L146K AAG D212Q CAG K279D GAT L345P CCT F014V GTG P080R AGG L146N AAT D212S TCG K279F TTT L345Q CAG F014W TGG P080S TCT L146P CCT D212T ACT K279G GGG L345R CGT L015A GCG P080T ACG L146Q CAG D212V GTG K279H CAT L345T ACT L015E GAG P080V GTG L146R CGG D212W TGG K279L CTG L345V GTT L015F TTT P080Y TAT L146S TCG D213A GCT K279P CCT L345W TGG L015G GGG Q081A GCT L146T ACT D213E GAG K279Q CAG L345Y TAT L015K AAG Q081C TGT L146V GTT D213G GGG K279R AGG C346A GCT L015M ATG Q081E GAG L146Y TAT D213H CAT K279S TCT C346D GAT L015N AAT Q081F TTT T147A GCT D213K AAG K279T ACG C346F TTT L015P CCG Q081G GGG T147C TGT D213L CTG K279V GTG C346G GGG L015Q CAG Q081H CAT T147D GAT D213M ATG K279W TGG C346I ATT L015R CGG Q081L CTG T147F TTT D213N AAT K279Y TAT C346K AAG L015S TCG Q081M ATG T147G GGT D213P CCT F280D GAT C346L CTT L015T ACT Q081N AAT T147I ATT D213Q CAG

F280E GAG C346M ATG L015V GTT Q081P CCG T147L CTT D213R CGT F280G GGG C346P CCT L015W TGG Q081R AGG T147M ATG D213S TCG F280H CAT C346Q CAG L015Y TAT Q081S TCT T147P CCT D213V GTG F280I ATT C346R CGG W016A GCG Q081V GTT T147Q CAG D213W TGG F280L TTG C346S TCT W016C TGT Q081W TGG T147R CGT D213Y TAT F280M ATG C346T ACT W016D GAT Q081Y TAT T147S AGT L214A GCG F280N AAT C346V GTG W016E GAG K082A GCT T147V GTT L214C TGT F280P CCT C346W TGG W016F TTT K082E GAG T147W TGG L214D GAT F280Q CAG Q347A GCT W016G GGT K082G GGT T147Y TAT L214E GAG F280R CGT Q347C TGT W016H CAT K082H CAT E148C TGT L214G GGG F280S TCG Q347E GAG W016K AAG K082I ATT E148F TTT L214H CAT F280T ACT Q347F TTT W016L CTT K082L CTT E148G GGG L214K AAG F280V GTG Q347G GGT W016M ATG K082M ATG E148H CAT L214N AAT F280W TGG Q347I ATT W016P CCT K082N AAT E148I ATT L214P CCG L281A GCG Q347L TTG W016R CGT K082P CCT E148K AAG L214Q CAG L281D GAT Q347M ATG W016S TCG K082Q CAG E148L CTG L214R CGG L281F TTT Q347P CCT W016T ACT K082R CGT E148P CCT L214S TCG L281G GGT Q347R AGG W016Y TAT K082S AGT E148Q CAG L214T ACG L281H CAT Q347S TCT A017D GAT K082T ACT E148R CGG L214V GTG L281I ATT Q347T ACT

A017E GAG K082V GTG E148S TCT L214Y TAT L281K AAG Q347V GTG A017G GGG K082W TGG E148T ACT S215A GCT L281N AAT Q347W TGG A017H CAT K082Y TAT E148V GTG S215C TGT L281P CCG Q347Y TAT A017I ATT I083E GAG E148W TGG S215D GAT L281Q CAG E348C TGT A017L CTT I083F TTT E148Y TAT S215E GAG L281R CGG E348D GAT A017N AAT I083G GGT A149C TGT S215G GGG L281S AGT E348G GGT A017P CCG I083H CAT A149E GAG S215H CAT L281V GTT E348H CAT A017Q CAG I083K AAG A149F TTT S215K AAG L281W TGG E348I ATT A017R AGG I083L CTG A149G GGT S215L TTG L281Y TAT E348L TTG A017S TCG I083N AAT A149K AAG S215M ATG S282A GCG E348M ATG A017T ACG I083P CCT A149L TTG S215P CCG S282C TGT E348P CCT A017V GTG I083Q CAA A149M ATG S215Q CAG S282D GAT E348Q CAG A017W TGG I083R CGT A149P CCT S215R CGG S282E GAG E348R CGG A017Y TAT I083S TCG A149Q CAG S215T ACT S282F TTT E348S TCT W018C TGT I083T ACT A149R CGG S215V GTG S282G GGT E348T ACT W018D GAT I083V GTT A149S TCT S215W TGG S282L CTT E348V GTT W018F TTT I083Y TAT A149T ACT W216D GAT S282M ATG E348W TGG W018G GGG S084D GAT A149V GTT W216E GAG S282P CCT E348Y TAT W018H CAT S084E GAG A149W TGG W216G GGT S282Q CAG Q349A GCT W018I ATT S084F TTT A149Y TAT W216H CAT S282R CGT Q349D GAT W018L CTG S084G GGT T150A GCT W216I ATT S282T ACT Q349E GAG W018M ATG S084H CAT T150C TGT W216K AAG S282V GTT Q349F TTT W018P CCG S084I ATT T150D GAT W216L CTG S282W TGG Q349G GGT W018Q CAG S084L CTT T150E GAG W216M ATG S282Y TAT Q349H CAT W018R CGG S084M ATG T150F TTT W216N AAT Q283A GCG Q349K AAG W018S AGT S084N AAT T150G GGG W216P CCT Q283C TGT Q349L CTG W018T ACG S084P CCT T150I ATT W216Q CAG Q283D GAT Q349M ATG W018V GTG S084Q CAG T150L TTG W216R CGG Q283E GAG Q349N AAT W018Y TAT S084R CGG T150N AAT W216T ACG Q283F TTT Q349P CCT N019A GCG S084T ACT T150P CCT W216V GTG Q283G GGG Q349R CGT N019C TGT S084W TGG T150R AGG W216Y TAT Q283H CAT Q349S TCG N019F TTT S084Y TAT T150S TCT L217A GCG Q283L CTT Q349T ACT N019G GGG L085A GCT T150V GTG L217C TGT Q283N AAT Q349V GTG N019H CAT L085C TGT T150W TGG L217E GAG Q283P CCG

Q349W TGG N019I ATT L085D GAT T150Y TAT L217G GGT Q283R CGT Q349Y TAT N019L CTG L085E GAG E151A GCT L217H CAT Q283S TCT G350A GCT N019M ATG L085F TTT E151C TGT L217I ATT Q283T ACT G350D GAT N019P CCG L085G GGG E151G GGT L217M ATG Q283W TGG G350E GAG N019Q CAG L085H CAT E151H CAT L217P CCG Q283Y TAT G350F TTT N019R CGT L085K AAG E151K AAG L217Q CAG D284A GCT G350H CAT N019S TCG L085N AAT E151L TTG L217R AGG D284C TGT G350K AAG N019V GTT L085P CCT E151M ATG L217S TCT D284E GAG G350L CTG N019W TGG L085Q CAG E151N AAT L217T ACG D284G GGT G350M ATG N019Y TAT L085R CGT E151Q CAG L217V GTG D284H CAT G350N AAT A020D GAT L085S TCG E151R AGG L217W TGG D284I ATT G350P CCT A020E GAG L085T ACT E151S TCG L217Y TAT D284L TTG G350R CGT A020F TTT L085V GTT E151T ACT W218A GCT D284M ATG G350S TCT A020G GGG Q086A GCT E151V GTT W218D GAT D284N AAT G350T ACT A020H CAT Q086C TGT E151W TGG W218F TTT D284P CCG G350V GTG A020K AAG Q086D GAT E151Y TAT W218G GGT D284Q CAG G350Y TAT A020L CTG Q086E GAG K152A GCT W218H CAT D284S TCT V351A GCT A020N AAT Q086F TTT K152C TGT W218I ATT D284T ACG V351C TGT A020P CCG Q086G GGT K152F TTT W218K AAG D284V GTT V351D GAT A020Q CAG Q086H CAT K152G GGT W218L CTT D284Y TAT V351E GAG A020R CGT Q086I ATT K152I ATT W218M ATG E285A GCG V351F TTT A020S TCT Q086K AAG K152L TTG W218P CCT E285F TTT V351G GGT A020T ACT Q086L CTG K152M ATG W218Q CAG E285G GGG V351H CAT A020V GTT Q086M ATG K152N AAT W218R CGG E285H CAT V351I ATT A020Y TAT Q086N AAT K152P CCT W218S TCG E285K AAG V351L TTG P021A GCG Q086P CCT K152R AGG W218T ACT E285M ATG V351N AAT P021C TGT Q086R CGG K152S TCT W218V GTG E285N AAT V351Q CAG P021D GAT Q086S TCT K152T ACT N219A GCG E285P CCT V351R AGG P021E GAG Q086T ACT K152V GTG N219C TGT E285Q CAG V351S TCT P021G GGG Q086V GTG K152W TGG N219D GAT E285R CGT V351W TGG P021H CAT Q086W TGG K152Y TAT N219E GAG E285S AGT V351Y TAT P021I ATT D087A GCT A153C TGT N219G GGG E285T ACG C352A GCT P021K AAG D087C TGT A153E GAG N219H CAT E285V GTG C352D GAT P021L CTT D087E GAG A153F TTT N219I ATT E285W TGG C352E GAG P021M ATG D087G GGG A153G GGT N219K AAG E285Y TAT C352F TTT P021R CGT D087H CAT A153H CAT N219L CTT L286A GCG C352G GGG P021S TCT D087I ATT A153I ATT N219M ATG L286C TGT C352K AAG P021T ACG D087L CTG A153K AAG N219P CCT L286D GAT C352M ATG P021V GTT D087M ATG A153L CTG N219R CGT L286E GAG C352P CCT P021W TGG D087P CCT A153M ATG N219S TCG L286F TTT C352Q CAG S022A GCT D087Q CAG A153P CCT N219T ACT L286G GGT C352R CGT S022C TGT D087R AGG A153Q CAG N219W TGG L286H CAT C352S AGT S022D GAT D087S TCG A153R CGT E220A GCG L286K AAG C352T ACT S022E GAG D087T ACT A153S AGT E220D GAT L286M ATG C352V GTG S022G GGG D087V GTT A153T ACT E220G GGG L286P CCT C352W TGG S022H CAT D087Y TAT A153V GTG E220H CAT L286R AGG C352Y TAT S022K AAG H088A GCT A153W TGG E220I ATT L286S AGT I353A GCT S022L CTG H088C TGT K154A GCT E220K AAG L286T ACG I353C TGT S022M ATG H088E GAG K154C TGT E220L TTG L286W TGG I353E GAG S022N AAT H088F TTT K154D GAT E220M ATG L286Y TAT I353F TTT S022P CCG H088G GGG K154E GAG E220N AAT V287A GCT I353G GGG S022R CGG H088I ATT K154G GGT E220P CCG V287C TGT I353H CAT S022T ACT H088K AAG K154H CAT E220R CGG V287D GAT I353K AAG S022V GTG H088L TTG K154I ATT

E220S TCT V287E GAG I353L CTT S022Y TAT H088M ATG K154L CTG E220T ACG V287F TTT I353M ATG E023A GCT H088P CCT K154P CCT E220V GTG V287G GGG I353Q CAG E023D GAT H088R CGT K154R CGG E220W TGG V287I ATT I353R CGT E023F TTT H088S AGT K154S AGT S221A GCG V287K AAG I353S TCG E023G GGG H088T ACT K154T ACT S221C TGT V287L CTT I353T ACT E023H CAT H088V GTT K154V GTG S221D GAT V287N AAT I353V GTG E023L CTT H088Y TAT K154W TGG S221E GAG V287P CCT I353W TGG E023M ATG L089A GCT K154Y TAT S221G GGG V287Q CAG R354C TGT E023N AAT L089C TGT Q155A GCT S221H CAT V287R CGG R354D GAT E023P CCT L089D GAT Q155C TGT S221I ATT V287S TCT R354E GAG E023Q CAG L089E GAG Q155D GAT S221K AAG V287T ACT R354G GGT E023R CGG L089G GGG Q155F TTT S221L TTG Y288D GAC R354H CAT E023S TCT L089K AAG Q155G GGG S221M ATG Y288E GAG R354I ATT E023T ACG L089M ATG Q155H CAT S221P CCG Y288F TTT R354K AAG E023V GTG L089N AAT Q155K AAG S221Q CAG Y288G GGG R354L CTT E023W TGG L089P CCT Q155L CTT S221R CGG Y288H CAT R354M ATG F024A GCG L089Q CAG Q155M ATG S221T ACT Y288I ATT R354P CCT F024C TGT L089R AGG Q155P CCT S221V GTG Y288K AAG R354Q CAG F024E GAG L089S TCG Q155R CGG T222A GCG Y288L CTG R354S TCT F024G GGG L089T ACT Q155S AGT T222D GAT Y288P CCT R354V GTG F024H CAT L089W TGG Q155T ACT T222E GAG Y288Q CAG R354W TGG F024I ATT L089Y TAT Q155V GTT T222F TTT Y288R CGT R354Y TAT F024K AAG D090A GCT Q155W TGG T222G GGG Y288S TCT K355D GAT F024L TTG D090C TGT Q155Y TAT T222I ATT Y288T ACT K355F TTT F024M ATG D090E GAG E156A GCT T222K AAA Y288V GTG K355G GGG F024N AAT D090G GGG E156C TGT T222L TTG Y288W TGG K355H CAT F024P CCT D090H CAT E156D GAT T222N AAT T289A GCT K355L CTG F024R CGT D090I ATT E156G GGT T222P CCG T289C TGT K355M ATG F024T ACG D090K AAG E156I ATT T222R CGG T289E GAG K355N AAT F024V GTT D090L CTT E156K AAG T222S AGT T289G GGT K355P CCT F024Y TAT D090N AAT E156L CTG T222V GTT T289H CAT K355Q CAG C025D GAT D090P CCT E156M ATG T222W TGG T289K AAG K355R CGT C025E GAG D090Q CAG E156P CCT T222Y TAT T289L CTT K355S TCT C025F TTT D090R AGG E156Q CAG A223C TGT T289M ATG K355T ACT C025G GGG D090S AGT E156R CGG A223D GAT T289N AAT K355V GTG C025H CAT D090T ACT E156S TCT A223E GAG T289P CCT K355W TGG C025I ATT D090W TGG E156T ACT A223G GGG T289Q CAG K355Y TAT C025K AAG K091A GCT E156V GTT A223H CAT T289R AGG N356A GCT C025L TTG K091D GAT E156W TGG A223K AAG T289S TCG N356C TGT C025N AAT K091E GAG F157A GCT A223L CTG T289V GTG N356D GAT C025P CCT K091F TTT F157C TGT A223P CCT T289Y TAT N356F TTT C025R CGT K091G GGG F157D GAT A223Q CAG F290A GCT N356G GGG C025S TCT K091H CAT F157E GAG A223R AGG F290C TGT N356H CAT C025T ACT K091I ATT F157G GGT A223S TCT F290D GAT N356K AAG C025V GTG K091L TTG F157H CAT A223T ACG F290G GGG N356L CTG C025Y TAT K091N AAT F157I ATT A223V GTG F290H CAT N356P CCT L026A GCT K091Q CAG F157K AAG A223W TGG F290I ATT N356Q CAG L026E GAG K091R CGT F157L TTG A223Y TAT F290K AAG N356R CGG L026G GGT K091S TCT F157M ATG L224A GCT F290L TTG N356S AGT L026H CAT K091T ACT F157P CCT L224D GAT F290M ATG N356T ACT L026I ATT K091Y TAT F157Q CAG L224E GAG F290Q CAG N356V GTG L026K AAG A092C TGT F157R CGG L224F TTT F290R AGG N356W TGG L026M ATG A092E GAG F157S TCG L224G GGG F290S TCG W357A GCT L026P CCG

A092F TTT F157T ACT L224I ATT F290T ACT W357C TGT L026Q CAG A092G GGT F157V GTG L224M ATG F290V GTT W357D GAT L026R CGG A092H CAT F157W TGG L224P CCG F290Y TAT W357E GAG L026S TCT A092K AAG E158A GCT L224Q CAG G291A GCT W357F TTT L026T ACT A092L CTG E158C TGT L224R AGG G291C TGT W357G GGG L026V GTT A092M ATG E158D GAT L224S AGT G291D GAT W357K AAG L026W TGG A092P CCT E158F TTT L224T ACT G291E GAG W357L TTG L026Y TAT A092Q CAG E158G GGG L224V GTT G291F TTT W357M ATG G027A GCT A092R CGT E158H CAT L224W TGG G291H CAT W357P CCT G027C TGT A092T ACT E158K AAG L224Y TAT G291L CTG W357Q CAG G027D GAT A092V GTT E158L CTG Y225A GCG G291M ATG W357R CGT G027E GAG A092W TGG E158N AAT Y225D GAT G291N AAT W357S AGT G027F TTT A092Y TAT E158P CCT Y225E GAG G291P CCT W357T ACT G027H CAT K093D GAT E158Q CAG Y225G GGT G291Q CAG W357V GTG G027I ATT K093E GAG E158R CGG Y225H CAT G291R CGG N358C TGT G027K AAG K093F TTT E158S TCG Y225K AAG G291S TCT N358D GAT G027L CTG K093G GGT E158V GTG Y225L CTG G291T ACT N358E GAG G027P CCT K093H CAT E158Y TAT Y225P CCG G291V GTG N358G GGG G027Q CAG K093I ATT K159A GCT Y225Q CAG G291W TGG N358H CAT G027R CGG K093L CTG K159D GAT Y225R AGG G291Y TAT N358I ATT G027S TCG K093M ATG K159E GAG Y225S TCT E292A GCT N358K AAG G027T ACT K093N AAT K159F TTT Y225T ACG E292C TGT N358L CTG G027W TGG K093P CCT K159G GGT Y225V GTG E292F TTT N358P CCT K028A GCG K093Q CAG K159H CAT Y225W TGG E292G GGT N358Q CAG K028D GAT K093R CGG K159L CTT P226A GCG E292H CAT N358R CGT K028E GAG K093S AGT K159M ATG P226C TGT E292I ATT N358S TCT K028F TTT K093T ACT K159N AAT P226D GAT E292K AAG N358T ACT K028G GGG K093V GTT K159Q CAG P226E GAG E292L TTG N358V GTG K028I ATT K094A GCT K159R CGG P226F TTT E292N AAT N358W TGG K028L TTG K094C TGT K159S TCT P226G GGT E292P CCT S359A GCT K028M ATG K094D GAT K159V GTG P226L CTT E292Q CAG S359C TGT K028N AAT K094E GAG K159W TGG P226N AAT E292R CGG S359D GAT K028P CCT K094F TTT K159Y TAT P226Q CAG E292T ACT S359E GAG K028R CGG K094G GGG A160C TGT P226R AGG E292V GTT S359F TTT K028S AGT K094H CAT A160F TTT P226S TCT E292W TGG S359G GGG K028T ACT K094L TTG A160G GGG P226T ACG T293A GCT S359H CAT K028V GTT K094M ATG A160H CAT P226V GTT T293C TGT S359K AAG K028W TGG K094N AAT A160I ATT P226W TGG T293D GAT S359L TTG F029A GCT K094P CCT A160K AAG P226Y TAT T293E GAG S359M ATG F029C TGT K094Q CAG A160L CTG S227A GCT T293F TTT S359P CCT F029E GAG K094R AGG A160M ATG S227F TTT T293G GGT S359R CGG F029G GGG K094S TCT A160N AAT S227G GGG T293K AAG S359T ACT F029H CAT K094T ACT A160Q CAG S227H CAT T293L CTT S359V GTT F029I ATT D095A GCT A160R AGG S227I ATT T293M ATG S359W TGG F029K AAG D095C TGT A160S AGT S227K AAG T293N AAT S360A GCT F029L CTT D095E GAG A160V GTG S227L TTG T293P CCT S360C TGT F029M ATG D095F TTT A160W TGG S227M ATG T293Q CAG S360E GAG F029P CCG D095G GGG A160Y TAT S227P CCT T293S TCT S360F TTT F029R CGG D095H CAT G161A GCT S227Q CAG T293V GTG S360G GGG F029S TCG D095K AAG G161C TGT S227R CGG T293Y TAT S360I ATT F029T ACG D095L TTG G161D GAT S227T ACG V294A GCT S360K AAG F029V GTG D095M ATG G161E GAG S227V GTG V294C TGT S360L CTG F029W TGG D095P CCT G161H CAT S227W TGG V294E GAG S360M ATG D030A GCG D095Q CAG G161I ATT S227Y TAT V294G GGG

S360N AAT D030E GAG D095S TCT G161K AAG I228A GCG V294H CAT S360P CCT D030F TTT D095V GTG G161L CTT I228E GAG V294K AAG S360Q CAG D030G GGG D095W TGG G161M ATG I228F TTT V294L TTG S360R AGG D030H CAT D095Y TAT G161Q CAG I228G GGG V294M ATG S360T ACT D030K AAG I096A GCT G161R CGT I228H CAT V294N AAT S360V GTT D030L TTG I096C TGT G161S AGT I228K AAG V294P CCT D361A GCT D030M ATG I096D GAT G161T ACT I228L TTG V294Q CAG D361C TGT D030P CCT I096E GAG G161V GTG I228M ATG V294R AGG D361E GAG D030Q CAG I096F TTT G161W TGG I228N AAT V294S AGT D361G GGG D030R CGG I096G GGG K162A GCT I228P CCG V294T ACT D361H CAT D030S TCG I096H CAT K162D GAT I228Q CAG V294W TGG D361L TTG D030T ACT I096L TTG K162E GAG I228R CGT A295C TGT D361M ATG D030V GTT I096N AAT K162F TTT I228S TCT A295D GAT D361N AAT D030W TGG I096P CCT K162G GGG I228T ACT A295E GAG D361P CCT E031A GCG I096R CGT K162H CAT I228W TGG A295F TTT D361Q CAG E031C TGT I096S AGT K162L TTG Y229E GAG A295G GGG D361R AGG E031G GGG I096T ACT K162M ATG Y229F TTT A295H CAT D361S TCG E031H CAT I096V GTG K162P CCT Y229G GGT A295I ATT D361V GTT E031I ATT I096W TGG K162Q CAG Y229H CAT A295L CTG D361W TGG E031K AAG T097A GCT K162R CGG Y229I ATT A295N AAT D361Y TAT E031L CTG T097C TGT K162S TCG Y229K AAG A295P CCT Y362A GCT E031N AAC T097D GAT K162V GTG Y229L TTG A295Q CAG Y362C TGT E031P CCG T097E GAG K162W TGG Y229N AAT A295S AGT Y362E GAG E031R CGG T097F TTT K162Y TAT Y229P CCT A295T ACT Y362G GGG E031S TCT T097G GGG D163A GCT Y229Q CAG A295V GTT Y362H CAT E031T ACG T097I ATT D163C TGT Y229R CGT A295Y TAT Y362K AAG E031V GTG T097L CTT D163E GAG Y229S TCG L296A GCT Y362L CTT E031W TGG T097N AAT D163F TTT Y229T ACT L296C TGT Y362M ATG E031Y TAT T097P CCT D163G GGG Y229V GTG L296F TTT Y362N AAT P032A GCG T097Q CAG D163H CAC Y229W TGG L296G GGT Y362P CCT P032C TGT T097R CGG D163K AAG L230A GCG L296I ATT Y362R CGG P032F TTT T097S TCG D163L CTT L230E GAG L296K AAG Y362S AGT P032G GGG T097W TGG D163P CCT L230G GGG L296M ATG Y362T ACT P032H CAT T097Y TAT D163Q CAG L230H CAT L296P CCT Y362V GTG P032K AAG F098A GCT D163R AGG L230I ATT L296Q CAG Y362W TGG P032L CTG F098C TGT D163S TCG L230K AAG L296R CGT L363A GCT P032M ATG F098D GAT D163T ACT L230M ATG L296S TCG L363C TGT P032N AAT F098E GAG D163V GTG L230N AAT L296T ACT L363D GAT P032Q CAG F098G GGG D163W TGG L230P CCT L296V GTT L363E GAG P032R CGG F098H CAT F164A GCT L230R CGT L296W TGG L363F TTT P032S TCG F098I ATT F164C TGT L230S AGT L296Y TAT L363G GGG P032T ACT F098L TTG F164D GAT L230T ACT G297A GCT L363H CAT P032V GTG F098M ATG F164E GAG L230V GTT G297C TGT L363I ATT P032W TGG F098P CCT F164G GGG L230W TGG G297E GAG L363P CCT P032Y TAT F098Q CAG F164H CAT L230Y TAT G297H CAT L363Q CAG L033C TGT F098R CGT F164L TTG N231A GCT G297I ATT L363R CGG L033D GAT F098S TCG F164M ATG N231C TGT G297L CTT L363S TCG L033G GGG F098V GTT F164N AAT N231D GAT G297N AAT L363T ACT L033H CAT F098W TGG F164P CCT N231F TTT G297P CCT L363V GTG L033I ATT Y099A GCT F164Q CAG N231G GGG G297Q CAG L363W TGG L033M ATG Y099C TGT F164R CGG N231H CAT G297R CGG H364A GCT L033N AAT Y099E GAG F164S AGT N231I ATT G297S AGT H364C TGT L033P CCG Y099F TTT F164V GTT N231K AAG G297T ACT H364D GAT L033Q CAG Y099G GGT F164W TGG

N231L CTT G297V GTG H364E GAG L033R AGG Y099I ATT L165A GCT N231P CCT G297W TGG H364F TTT L033S TCG Y099L TTG L165C TGT N231Q CAG G297Y TAT H364G GGG L033T ACT Y099N AAT L165D GAT N231R CGT A298C TGT H364K AAG L033V GTT Y099P CCT L165F TTT N231S TCT A298E GAG H364L CTG L033W TGG Y099Q CAG L165G GGG N231T ACG A298G GGG H364M ATG L033Y TAT Y099R AGG L165H CAT N231V GTG A298I ATT H364P CCT D034A GCT Y099S TCG L165N AAT T232A GCG A298L TTG H364R CGG D034E GAG Y099T ACT L165P CCT T232C TGT A298M ATG H364S TCT D034G GGT Y099V GTT L165Q CAG T232F TTT A298N AAT H364T ACT D034H CAT Y099W TGG L165R CGG T232G GGG A298P CCT H364V GTG D034I ATT M100C TGT L165S TCG T232H CAT A298Q CAG H364Y TAT D034K AAG M100E GAG L165T ACT T232K AAG A298R CGT L365A GCT D034L CTT M100F TTT L165V GTG T232L CTT A298S TCG L365C TGT D034N AAT M100G GGT L165W TGG T232M ATG A298T ACT L365D GAT D034P CCT M100K AAG L165Y TAT T232N AAT A298V GTG L365E GAG D034Q CAG M100L CTG V166A GCT T232P CCG A298W TGG L365G GGG D034R CGT M100N AAT V166C TGT T232Q CAG A298Y TAT L365I ATT D034S AGT M100P CCT V166D GAT T232R AGG S299A GCT L365M ATG D034T ACG M100Q CAG V166E GAG T232S AGT S299C TGT L365N AAT D034V GTT M100R CGG V166F TTT T232V GTG S299D GAT L365P CCT D034W TGG M100S TCT V166G GGT T232Y TAT S299E GAG L365Q CAG M035A GCG M100T ACT V166H CAT Q233A GCG S299F TTT L365R CGG M035D GAT M100V GTT V166L CTT Q233C TGT S299G GGG L365S AGT M035F TTT M100W TGG V166N AAT Q233D GAT S299H CAT L365T ACT M035G GGG M100Y TAT V166P CCT Q233F TTT S299I ATT L365V GTG M035H CAT P101A GCT V166Q CAG Q233G GGG S299L CTT L365W TGG M035I ATT P101C TGT V166R CGG Q233I ATT S299M ATG L365Y TAT M035L TTG P101F TTT V166T ACT Q233K AAG S299P CCT N366A GCT M035N AAT P101G GGG V166W TGG Q233L CTG S299Q CAG N366C TGT M035P CCG P101H CAT V166Y TAT Q233P CCG S299R AGG N366E GAG M035Q CAG P101I ATT E167A GCT Q233R AGG S299T ACT N366F TTT M035R CGT P101K AAG E167D GAT Q233S TCG S299Y TAT N366G GGG M035S TCT P101L CTT E167F TTT Q233T ACG G300A GCT N366K AAG M035T ACT P101M ATG E167G GGT Q233V GTG G300C TGT N366L TTG M035V GTT P101N AAT E167H CAT Q233W TGG G300D GAT N366M ATG M035Y TAT P101Q CAG E167K AAG Q233Y TAT G300E GAG N366P CCT S036A GCG P101R AGG E167L TTG Q234A GCT G300F TTT N366Q CAG S036C TGT P101S TCT E167M ATG Q234C TGT G300L CTT N366R AGG S036D GAT P101T ACT E167N AAT Q234D GAT G300M ATG N366S TCT S036F TTT P101Y TAT E167P CCT Q234E GAG G300N AAT N366T ACT S036G GGT V102A GCT E167R AGG Q234G GGT G300P CCT N366V GTT S036H CAT V102C TGT E167S TCG Q234H CAT G300Q CAG N366W TGG S036K AAG V102E GAG E167T ACT Q234L CTT G300R AGG P367A GCT S036L TTG V102G GGT E167V GTT Q234M ATG G300S TCG P367C TGT S036N AAT V102H CAT E167Y TAT Q234N AAT G300T ACT P367E GAG S036P CCG V102K AAG T168A GCT Q234P CCG G300V GTT P367F TTT S036R CGG V102L TTG T168C TGT Q234R CGG G300W TGG P367G GGT S036T ACG V102M ATG T168D GAT Q234S AGT I301A GCT P367H CAT S036V GTT V102N AAT T168E GAG Q234T ACT I301E GAG ~~P367I~~P387I ATT S036W TGG V102P CCT T168F TTT Q234V GTG I301G GGG P367K AAG S036Y TAT V102Q CAG T168G GGG Q234W TGG I301H CAT P367L CTG L037A GCG V102R AGG T168H CAT S235A GCG I301K AAG P367M ATG L037C TGT V102S TCT T168K AAG S235E GAG I301L CTG P367Q CAG L037E GAG

V102T ACT T168L CTG S235F TTT I301M ATG P367R CGT L037F TTT V102W TGG T168P CCT S235G GGG I301N AAT P367S TCG L037G GGG D103A GCT T168R CGG S235H CAT I301P CCT P367V GTT L037I ATT D103E GAG T168S TCT S235K AAG I301Q CAG P367W TGG L037K AAG D103F TTT T168V GTG S235L CTT I301R CGG D368A GCT L037M ATG D103G GGG T168W TGG S235M ATG I301S AGT D368C TGT L037N AAT D103H CAT T168Y TAT S235P CCT I301V GTT D368E GAG L037P CCT D103I ATT I169A GCT S235Q CAG I301W TGG D368G GGT L037R AGG D103L CTT I169D GAT S235R CGG I301Y TAT D368H CAT L037S TCT D103N AAT I169F TTT S235T ACG V302C TGT D368K AAG L037T ACG D103Q CAG I169G GGG S235V GTG V302D GAT D368L CTT L037V GTG D103R AGG I169H CAT S235W TGG V302E GAG D368M ATG L037W TGG D103S TCG I169K AAG S235Y TAT V302F TTT D368P CCT F038A GCG D103T ACT I169L TTG P236A GCT V302G GGT D368R CGT F038C TGT D103V GTT I169N AAT P236C TGT V302H CAT D368S AGT F038E GAG D103W TGG I169P CCT P236E GAG V302I ATT D368T ACT F038G GGG D103Y TAT I169Q CAG P236G GGG V302L TTG D368V GTT F038K AAG N104A GCT I169R CGG P236H CAT V302M ATG D368W TGG F038L CTT N104C TGT I169S TCG P236I ATT V302P CCT D368Y TAT F038M ATG N104F TTT I169T ACT P236K AAG V302R AGG N369A GCT F038N AAT N104G GGG I169V GTT P236L CTG V302S TCG N369C TGT F038P CCT N104H CAT I169Y TAT P236N AAT V302T ACT N369E GAG F038Q CAG N104I ATT K170A GCT P236Q CAG V302W TGG N369F TTT F038R AGG N104K AAG K170C TGT P236R CGT V302Y TAT N369H CAT F038S TCT N104L CTG K170D GAT P236S AGT I303A GCT N369I ATT F038T ACT N104M ATG K170E GAG P236T ACT I303C TGT N369K AAG F038W TGG N104P CCT K170G GGG P236W TGG I303D GAT N369L CTT F038Y TAT N104R AGG K170I ATT P236Y TAT I303E GAG N369P CCT S039A GCG N104S TCT K170L TTG V237A GCG I303F TTT N369Q CAG S039C TGT N104T ACT K170M ATG V237C TGT I303G GGT N369R CGG S039D GAT N104V GTT K170N AAT V237E GAG I303K AAG N369S TCG S039F TTT N104W TGG K170P CCT V237F TTT I303L TTG N369T ACT S039G GGT L105A GCT K170Q CAG V237G GGT I303M ATG N369V GTG S039L TTG L105C TGT K170R CGT V237H CAT I303P CCT N369W TGG S039M ATG L105D GAT K170V GTT V237L TTG I303R CGT F370A GCT S039N AAT L105E GAG K170W TGG V237N AAT I303S AGT F370D GAT S039P CCG L105G GGT K170Y TAT V237P CCT I303V GTG F370E GAG S039Q CAG L105H CAT L171A GCT V237Q CAG I303W TGG F370G GGG S039R CGT L105I ATT L171C TGT V237R CGG I303Y TAT F370H CAT S039T ACT L105M ATG L171D GAT V237S TCG W304A GCT F370I ATT S039V GTT L105N AAT L171G GGG V237T ACG W304C TGT F370K AAG S039W TGG L105P CCT L171H CAT V237W TGG W304D GAT F370L CTG S039Y TAT L105Q CAG L171I ATT V237Y TAT W304G GGT F370N AAT F040A GCG L105R CGG L171M ATG A238D GAT W304I ATT F370P CCT F040D GAT L105S TCT L171N AAT A238E GAG W304L CTG F370Q CAG F040E GAG L105T ACT L171P CCT A238F TTT W304M ATG F370R AGG F040G GGT L105V GTT L171Q CAG A238G GGT W304N AAT F370S TCT F040I ATT L105W TGG L171R CGT A238H CAT W304P CCT F370V GTG F040K AAG G106A GCT L171S AGT A238K AAG W304Q CAG F370Y TAT F040L CTG G106C TGT L171V GTG A238L CTT W304R CGG A371C TGT F040N AAT G106D GAT L171W TGG A238P CCG W304S AGT A371E GAG F040Q CAG G106E GAG L171Y TAT A238Q CAG W304T ACT A371F TTT F040R CGG G106F TTT G172A GCT A238R AGG W304V GTG A371G GGG F040S TCT G106H CAT G172C TGT A238S AGT W304Y TAT A371H CAT

F040T ACT G106I ATT G172D GAT A238T ACG G305C TGT A371I ATT F040V GTT G106L CTG G172E GAG A238V GTG G305D GAT A371K AAG F040W TGG G106M ATG G172I ATT A238W TGG G305E GAG A371L CTT F040Y TAT G106N AAT G172L CTT A238Y TAT G305F TTT A371M ATG I041A GCG G106P CCT G172M ATG A239C TGT G305H CAT A371P CCT I041C TGT G106S AGT G172P CCT A239F TTT G305K AAG A371R CGT I041D GAT G106V GTG G172Q CAG A239G GGT G305L CTT A371S TCG I041E GAG G106W TGG G172R CGT A239H CAT G305N AAT A371T ACT I041F TTT G106Y TAT G172S TCT A239I ATT G305P CCT A371V GTG I041G GGG M107A GCT G172T ACT A239K AAG G305Q CAG A371W TGG I041H CAT M107C TGT G172V GTT T240K AAG G305R CGT I372A GCT I041N AAT M107D GAT G172W TGG A239L TTG G305S TCG I372D GAT I041P CCG M107F TTT G172Y TAT A239N AAT G305T ACT I372E GAG I041Q CAG M107G GGG K173D GAT A239P CCT G305V GTG I372F TTT I041R AGG M107H CAT K173E GAG A239R AGG G305Y TAT I372G GGT I041S TCT M107I ATT K173G GGG A239S TCT T306A GCT I372H CAT I041T ACG M107K AAG K173H CAT A239T ACT T306C TGT I372K AAG I041V GTT M107L CTT K173I ATT A239V GTT T306D GAT I372L CTG I041W TGG M107P CCT K173L CTT A239W TGG T306E GAG I372N AAT G042A GCT M107Q CAG K173M ATG A239Y TAT T306F TTT I372P CCT G042C TGT M107R CGT K173N AAT T240A GCG T306G GGT I372R CGG G042D GAT M107S TCT K173P CCT T240E GAG T306H CAT I372S TCT G042E GAG M107V GTT K173Q CAG T240F TTT T306I ATT I372T ACT G042H CAT M107W TGG K173R CGG T240G GGG T306L CTG I372V GTG G042I ATT A108D GAT K173S TCG T240L CTT T306P CCT I372W TGG G042K AAG A108E GAG K173V GTG T240M ATG T306R AGG Q373A GCT G042L CTG A108F TTT K173W TGG T240N AAT T306S AGT Q373C TGT G042M ATG A108G GGT K173Y TAT T240P CCT T306V GTG Q373E GAG G042P CCT A108H CAT L174A GCT T240Q CAG T306W TGG Q373F TTT G042Q CAG A108K AAG L174C TGT T240R CGT T306Y TAT Q373G GGT G042R CGG A108L TTG L174G GGG T240S AGT L307C TGT Q373H CAT G042S TCT A108M ATG L174H CAT T240V GTG L307E GAG Q373K AAG G042T ACT A108N AAT L174K AAG T240W TGG L307F TTT Q373L CTG G042V GTT A108P CCT L174M ATG T240Y TAT L307G GGG Q373M ATG S043A GCG A108Q CAG L174N AAT L241A GCG L307I ATT Q373N AAT S043D GAT A108R CGG L174P CCT L241C TGT L307K AAG Q373P CCT S043E GAG A108S TCT L174Q CAG L241D GAT L307N AAT Q373R CGT S043F TTT A108T ACT L174R CGT L241E GAG L307P CCT Q373S TCT S043G GGT A108V GTG L174S TCG L241F TTT L307Q CAG Q373T ACT S043H CAT A108Y TAT L174T ACT L241G GGG L307R AGG Q373V GTT S043I ATT V109A GCT L174V GTT L241I ATT L307S AGT Q373W TGG S043K AAG V109C TGT L174W TGG L241K AAG L307T ACT L374A GCT S043L CTT V109D GAT L174Y TAT L241P CCT L307V GTG L374D GAT S043N AAT V109E GAG L175C TGT L241Q CAG L307W TGG L374E GAG S043P CCT V109F TTT L175D GAT L241R CGG L307Y TAT L374G GGT S043Q CAG V109G GGG L175E GAG L241S TCT S308C TGT L374H CAT S043R CGG V109H CAT L175F TTT L241T ACG S308D GAT L374I ATT S043T ACT V109L TTG L175G GGG L241V GTT S308F TTT L374M ATG S043V GTG V109M ATG L175H CAT L241W TGG S308G GGT L374N AAT P044A GCT V109P CCT L175K AAG Y242A GCG S308H CAT L374P CCT P044C TGT V109Q CAG L175N AAT Y242C TGT S308K AAG L374R AGG P044E GAG V109R AGG L175P CCT Y242D GAT S308L CTG L374S AGT P044F TTT V109T ACT L175R CGT Y242F TTT S308M ATG L374T ACT P044G GGG V109W TGG L175S TCT Y242G GGT

S308N AAT L374V GTG P044H CAT V109Y TAT L175T ACT Y242I ATT S308P CCT L374W TGG P044I ATT I110A GCT L175V GTG Y242K AAG S308R CGG L374Y TAT P044L CTT I110C TGT L175W TGG Y242L CTT S308T ACT E375A GCT P044N AAT I110D GAT L175Y TAT Y242M ATG S308V GTT E375C TGT P044Q CAG I110F TTT R176A GCT Y242P CCG S308W TGG E375F TTT P044R CGT I110G GGG R176C TGT Y242R CGG S308Y TAT E375G GGT P044S TCT I110H CAT R176E GAG Y242S TCT I309D GAT E375I ATT P044T ACT I110K AAG R176F TTT Y242T ACG I309E GAG E375K AAG P044W TGG I110L CTG R176G GGG Y242V GTT I309G GGT E375L CTT P044Y ACG I110M ATG R176H CAT Y242W TGG I309H CAT E375M ATG R045A GCG I110N AAT R176I ATT V243A GCG I309K AAG E375N AAT R045D GAT I110P CCT R176K AAG V243C TGT I309L CTG E375P CCT R045F TTT I110R CGT R176L CTT V243D GAT I309M ATG E375R CGT R045G GGG I110S AGT R176P CCT V243F TTT I309N AAT E375S TCT R045H CAT I110V GTT R176Q CAG V243G GGG I309Q CAG E375T ACT R045I ATT I110W TGG R176S AGT V243H CAT I309R CGT E375V GTT R045K AAG D111C TGT R176T ACT V243L CTT I309S AGT E375Y TAT R045M ATG D111E GAG R176V GTG V243M ATG I309T ACT K376A GCT R045P CCT D111G GGT R176W TGG V243P CCT I309V GTG K376D GAT R045Q CAG D111H CAT P177A GCT V243Q CAG I309W TGG K376E GAG R045S TCG D111I ATT P177C TGT V243R AGG I309Y TAT K376G GGG R045T ACG D111K AAG P177D GAT V243S AGT M310A GCT K376I ATT R045V GTG D111L TTG P177F TTT V243T ACG M310C TGT K376L TTG R045W TGG D111M ATG P177G GGG V243W TGG M310E GAG K376M ATG R045Y TAT D111P ACT P177H CAT V243Y TAT M310F TTT K376P CCT I046A GCG D111Q CAG P177L CTT R244A GCG M310G GGG K376Q CAG I046C TGT D111R CGG P177M ATG R244D GAT M310K AAG K376R CGT I046E GAG D111S AGT P177Q CAG R244G GGG M310L CTG K376S AGT I046F TTT D111T ACT P177R CGG R244H CAT M310N AAT K376T ACT I046H CAT D111V GTT P177S TCT R244I ATT M310P CCT K376V GTG I046L CTT D111W TGG P177T ACT R244K AAG M310Q CAG K376W TGG I046M ATG D111Y TAT P177V GTT R244M ATG M310R CGG K376Y TAT I046N AAT W112C TGT P177W TGG R244N AAT M310S AGT G377C TGT I046P CCT W112D GAT P177Y TAT R244P CCT M310V GTG G377D GAT I046R CGT W112E GAG N178A GCT R244Q CAG M310W TGG G377E GAG I046S TCT W112F TTT N178D GAT R244S TCT M310Y TAT G377F TTT I046T ACT W112G GGG N178E GAG R244T ACG R311A GCT G377H CAT I046V GTT W112H CAT N178G GGG R244V GTG R311C TGT G377I ATT I046W TGG W112I ATT N178I ATT R244W TGG R311E GAG G377K AAG I046Y TAT W112L CTT N178K AAG R244Y TAT R311F TTT G377L CTT N047A GCT W112N AAT N178L TTG N245A GCG R311G GGT G377M ATG N047D GAT W112P CCT N178M ATG N245C TGT R311H CAT G377P CCT N047F TTT W112Q CAG N178P CCT N245F TTT R311I ATT G377R AGG N047G GGG W112R CGT N178R CGG N245G GGG R311K AAG G377S TCG N047H CAT W112S TCT N178S AGT N245H CAT R311L TTG G377T ACT N047I ATT W112V GTT N178T ACT N245I ATT R311P CCT G377V GTG N047K AAG W112Y TAT N178V GTG N245K AAG R311Q CAG G377Y TAT N047L CTT E113A GCT N178W TGG N245L CTG R311S TCT G378D GAT N047M ATG E113C TGT N178Y TAT N245P CCG R311T ACT G378E GAG N047P CCT E113D GAT H179A GCT N245Q CAG R311V GTG G378F TTT N047Q CAG E113F TTT H179C TGT N245R CGG R311W TGG G378I ATT N047R CGG E113G GGG H179E GAG N245S TCG S312A GCT G378K AAG N047S TCT E113H CAT H179G GGG N245T ACG S312C TGT G378L CTG N047T ACG E113L CTT H179I ATT N245V GTG

S312E GAG G378M ATG N047V GTG E113P CCT H179K AAG N245W TGG S312F TTT G378N AAT N047W TGG E113Q CAG H179L CTG R246A GCG S312G GGG G378Q CAG N047Y TAT E113R CGT H179M ATG R246C TGT S312H CAT G378R AGG A048C TGT E113S TCT H179N AAT R246D GAT S312K AAG G378S TCT A048E GAG E113T ACT H179P CCT R246E GAG S312L CTG G378T ACT A048F TTT E113V GTT H179R AGG R246G GGG S312M ATG G378V GTG A048G GGT E113W TGG H179S AGT R246H CAT S312N AAT G378W TGG A048H CAT E113Y CAT H179T ACT R246I ATT S312P CCT G378Y TAT A048I ATT E114A GCT H179V GTG R246K AAG S312Q CAG K379A GCT A048K AAG E114C TGT H179W TGG R246L TTG S312R CGG K379C TGT A048L CTG E114D GAT L180A GCT R246M ATG S312T ACT K379E GAG A048M ATG E114G GGG L180C TGT R246P CCT S312V GTT K379F TTT A048N AAT E114H CAT L180E GAG R246S AGT S312W TGG K379G GGG A048P CCT E114I ATT L180F TTT R246T ACG M313A GCT K379H CAT A048Q CAG E114L CTG L180G GGT R246V GTT M313C TGT K379I ATT A048R CGG E114M ATG L180H CAT R246W TGG M313D GAT K379L CTT A048S TCT E114P CCT L180I ATT V247A GCG M313E GAG K379M ATG A048V GTT E114R CGG L180K AAG V247C TGT M313F TTT K379N AAT A048W TGG E114S TCT L180M ATG V247F TTT M313G GGG K379R CGT A048Y TAT E114T ACT L180N AAT V247H CAT M313H CAT K379S TCT T049A GCG E114V GTG L180P CCT V247I ATT M313K AAG K379T ACT T049C TGT E114W TGG L180R AGG V247L CTG M313L CTT K379V GTT T049D GAT E114Y TAT L180S TCG V247M ATG M313P CCT K379W TGG T049F TTT W115A GCT L180T ACT V247N AAT M313R CGT F380A GCT T049G GGG W115C TGT L180W TGG V247P CCT M313S TCG F380C TGT T049H CAT W115D GAT W181A GCT V247Q CAG M313T ACT F380D GAT T049I ATT W115F TTT W181C TGT V247R CGT M313V GTT F380E GAG T049K AAG W115G GGT W181D GAT V247S TCT M313Y TAT F380G GGG T049L TTG W115H CAT W181E GAG V247T ACT K314A GCT F380I ATT T049N AAT W115I ATT W181F TTT V247W TGG K314C TGT F380L CTT T049P CCG W115K AAG W181H CAT V247Y TAT K314D GAT F380P CCT T049R AGG W115L CTT W181I ATT R248A GCT K314H CAT F380Q CAG T049S TCG W115M ATG W181K AAG R248C TGT K314I ATT F380R CGG T049V GTT W115P CCT W181L CTG R248D GAT K314L TTG F380S AGT T049W TGG W115R CGG W181M ATG R248E GAG K314N AAT F380T ACT G050A GCG W115S AGT W181N AAT R248G GGG K314P CCT F380V GTG G050C TGT W115V GTG W181Q CAG R248H CAT K314Q CAG F380W TGG G050D GAT W115Y TAT W181R CGT R248I ATT K314R CGG F380Y TAT G050E GAG R116A GCT W181S TCT R248L CTT K314S TCG T381A AGC G050F TTT R116C TGT W181V GTG R248M ATG K314T ACT T381E GAG G050H CAT R116D GAT G182A GCT R248P CCG K314V GTT T381F TTT G050L CTT R116E GAG G182C TGT R248S TCG K314W TGG T381G GGT G050M ATG R116G GGG G182D GAT R248T ACG K314Y TAT T381H CAT G050P CCT R116H CAT G182E GAG R248V GTG S315A GCT T381K AAG G050Q CAG R116I ATT G182H CAT R248W TGG S315C TGT T381L TTG G050R CGG R116L CTG G182L CTT R248Y TAT S315E GAG T381N AAT G050S AGT R116N AAT G182M ATG E249A GCT S315G GGT T381P CCT G050V GTT R116P CCT G182N AAT E249G GGG S315H CAT T381Q CAG G050W TGG R116Q CAG G182P CCT E249H CAT S315I ATT T381R CGT G050Y TAT R116S TCT G182Q CAG E249I ATT S315K AAG T381S AGT Q051A GCG R116T ACT G182R CGT E249K AAG S315L CTG T381V GTG Q051C TGT R116V GTG G182S AGT E249L CTG S315M ATG T381W TGG Q051D GAT R116W TGG G182T ACT E249M ATG S315P CCT T381Y TAT Q051F TTT P117D GAT

G182V GTT E249P CCT S315R CGG V382E GAG Q051H CAT P117E GAG G182Y TAT E249Q CAG S315T ACT V382G GGG Q051I ATT P117F TTT Y183A GCT E249R CGG S315V GTT V382H CAT Q051K AAG P117G GGT Y183C TGT E249S TCT S315W TGG V382I ATT Q051M ATG P117H CAT Y183D GAT E249T ACT S315Y TAT V382K AAG Q051N AAT P117I ATT Y183E GAG E249V GTG C316A GCT V382L TTG Q051P CCT P117K AAG Y183G GGG E249W TGG C316D GAT V382M ATG Q051R CGG P117N AAT Y183I ATT E249Y TAT C316E GAG V382N AAT Q051S TCT P117Q CAG Y183K AAG A250C TGT C316G GGG V382P CCT Q051T ACG P117R AGG Y183L TTG A250F TTT C316I ATT V382Q CAG Q051W TGG P117S TCG Y183N AAT A250G GGT C316K AAG V382R CGG Q051Y TAT P117T ACT Y183P CCT A250H CAT C316L CTG V382S TCG G052A GCT P117V GTT Y183Q CAG A250K AAG C316M ATG V382T ACT G052C TGT P117W TGG Y183R CGT A250L CTG C316P CCT V382W TGG G052E GAG P117Y TAT Y183S TCT A250M ATG C316R AGG V382Y TAT G052F TTT T118C TGT Y183V GTT A250N AAT C316S TCT R383A GCT G052H CAT T118D GAT Y183W TGG A250P CCT C316T ACT R383E GAG G052K AAG T118E GAG Y184A GCT A250Q CAG C316V GTT R383F TTT G052L CTT T118G GGG Y184C TGT A250R AGG C316W TGG R383G GGG G052N AAT T118H CAT Y184D GAT A250S TCT C316Y TAT R383H CAT G052P CCT T118K AAG Y184E GAG A250T ACG L317A GCT R383I ATT G052Q CAG T118L CTG Y184F TTT A250V GTG L317C TGT R383K AAG G052R CGG T118M ATG Y184G GGT A250W TGG L317D GAT R383L CTG G052S AGT T118N AAT Y184H CAT I251C TGT L317G GGG R383M ATG G052T ACT T118P CCT Y184K AAG I251D GAT L317H CAT R383N AAT G052W TGG T118Q CAG Y184L CTT I251F TTT L317I ATT R383P CCT G052Y TAT T118R CGT Y184M ATG I251G GGG L317K AAG R383S TCG V053A GCG T118V GTT Y184P CCT I251H CAT L317M ATG R383T ACT V053C TGT T118W TGG Y184R AGG I251K AAG L317N AAT R383V GTG V053D GAT T118Y TAT Y184S TCG I251L CTT L317P CCT R383W TGG V053E GAG W119A GCT Y184V GTG I251M ATG L317Q CAG G384A GCT V053G GGG W119D GAT Y184W TGG I251P CCG L317R AGG G384C TGT V053H CAT W119E GAG L185A GCT I251Q CAG L317S TCG G384D GAT V053L CTG W119F TTT L185D GAT I251S AGT L317T ACT G384E GAG V053N AAT W119G GGT L185E GAG I251T ACT L317W TGG G384F TTT V053P CCG ~~W119I~~W1191 ATT L185F TTT I251V GTG L318C TGT G384H CAT V053Q CAG W119K AAG L185G GGG I251W TGG L318D GAT G384I ATT V053R CGG W119L CTG L185I ATT I251Y TAT L318F TTT G384K AAG V053S AGT W119N AAT L185K AAG R252A GCT L318G GGG G384L CTT V053T ACT W119P CCT L185N AAT R252D GAT L318H CAT G384M ATG V053W TGG W119Q CAG L185P CCT R252E GAG L318I ATT G384P CCT V053Y TAT W119R CGG L185R CGG R252F TTT L318K AAG G384Q CAG T054A GCG W119S TCT L185S TCG R252G GGT L318M ATG G384R AGG T054D GAT W119V GTT L185T ACT R252H CAT L318N AAT G384S TCG T054E GAG W119Y TAT L185V GTG R252I ATT L318P CCT G384T ACT T054F TTT A120C TGT L185W TGG R252K AAG L318Q CAG K385A GCT T054G GGG A120D GAT L185Y TAT R252L CTG L318R CGG K385C TGT T054H CAT A120F TTT F186A GCT R252N AAT L318S AGT K385G GGG T054I ATT A120G GGG F186D GAT R252P CCT L318T ACT K385H CAT T054M ATG A120H CAT F186G GGT R252S TCG L318W TGG K385L CTT T054N AAT A120I ATT F186H CAT R252T ACT L319C TGT K385M ATG T054P CCG A120L CTT F186I ATT R252V GTG L319E GAG K385N AAT T054Q CAG A120N AAT F186K AAG R252Y TAT L319F TTT K385P CCG T054R CGT A120P CCT F186L CTT V253A GCG L319G GGG K385Q CAG

T054S AGT A120R CGT F186N AAT V253D GAT L319H CAT K385R CGT T054V GTT A120S TCT F186P CCT V253E GAG L319I ATT K385S TCT T054Y TAT A120T ACT F186Q CAG V253G GGG L319K AAG K385T ACG I055A GCT A120V GTG F186R AGG V253H CAT L319M ATG K385V GTT I055C TGT A120W TGG F186S TCT V253I ATT L319P CCT K385W TGG I055D GAT A120Y TAT F186V GTT V253L CTG L319Q CAG K385Y TAT I055F TTT R121A GCT F186W TGG V253M ATG L319R AGG P386A GCG I055G GGG R121C TGT F186Y TAT V253N AAT L319S TCG P386C TGT I055H CAT R121D GAT P187A GCT V253P CCT L319V GTT P386F TTT I055L CTG R121E GAG P187F TTT V253Q CAG L319W TGG P386G GGG I055N AAT R121F TTT P187G GGG V253R CGG L319Y TAT P386H CAT I055P CCT R121G GGT P187H CAT V253S TCG D320C TGT P386I ATT I055Q CAG R121H CAT P187I ATT V253T ACG D320E GAG P386L CTT I055R CGT R121K AAG P187L CTT V253W TGG D320F TTT P386M ATG I055S TCG R121L CTG P187M ATG S254C TGT D320G GGG P386N AAT I055T ACT R121M ATG P187N AAT S254D GAT D320H CAT P386Q CAG I055V GTT R121P CCT P187Q CAG S254E GAG D320I ATT P386R CGT I055Y TAT R121S TCG P187R AGG S254G GGG D320K AAG P386S AGT F056A GCG R121T ACT P187S TCG S254I ATT D320L TTG P386T ACG F056C TGT R121V GTT P187T ACT S254K AAG D320M ATG P386V GTT F056E GAG R121W TGG P187V GTT S254L TTG D320N AAT P386Y TAT F056G GGG R121Y TAT P187W TGG S254N AAT D320P CCT T387C TGT F056H CAT N122A GCT P187Y TAT S254P CCT D320R AGG T387E GAG F056I ATT N122C TGT D188A GCT S254Q CAG D320S AGT T387F TTT F056K AAG N122E GAG D188C TGT S254R CGG D320V GTG T387G GGG F056L TTG N122F TTT D188F TTT S254T ACT D320W TGG T387H CAT F056N AAT N122I ATT D188G GGG S254V GTG D320Y TAT T387I ATT F056P CCG N122K AAG D188H CAT S254W TGG N321A GCT T387K AAG F056R CGT N122L CTG D188L CTT S254Y TAT N321D GAT T387L CTG F056S TCT N122M ATG D188M ATG K255A GCG N321E GAG T387M ATG F056T ACT N122P CCT D188N AAT K255C TGT N321G GGT T387N AAT F056V GTT N122Q CAG D188P CCT K255D GAT N321H CAT T387Q CAG F056W TGG N122R CGG D188Q CAG K255G GGT N321I ATT T387S TCG Y057A GCT N122S TCT D188R AGG K255H CAT N321K AAG T387V GTT Y057D GAT N122T ACT D188S AGT K255L TTG N321L CTG T387W TGG Y057E GAG N122V GTT D188T ACT K255N AAT N321M ATG T387Y TAT Y057F TTT N122W TGG D188V GTG K255P CCG N321P CCT L388A GCG Y057G GGG W123A GCT D188W TGG K255Q CAG N321R CGG L388C TGT Y057I ATT W123C TGT C189A GCT K255R CGG N321S TCT L388F TTT Y057L TTG W123D GAT C189E GAG K255S TCG N321T ACT L388G GGG Y057M ATG W123E GAG C189G GGT K255T ACT N321V GTG L388H CAT Y057P CCG W123G GGG C189H CAT K255V GTT N321Y TAT L388I ATT Y057Q CAG W123H CAT C189K AAG K255W TGG Y322C TGT L388M ATG Y057R CGG W123L CTT C189L TTG K255Y TAT Y322D GAT L388P CCT Y057S AGT W123M ATG C189M ATG I256A GCT Y322E GAG L388Q CAG Y057T ACG W123P CCT C189N ACT I256C TGT Y322F TTT L388R CGT Y057V GTG W123Q CAG C189P CCT I256D GAT Y322G GGT L388S TCG Y057W TGG W123R AGG C189R AGG I256E GAG Y322H CAT L388T ACG V058A GCT W123S AGT C189S TCG I256G GGG Y322I ATT L388V GTT V058C TGT W123T ACT C189T ACT I256H CAT Y322L CTG L388W TGG V058D GAT W123V GTT C189V GTG I256L CTT Y322N AAT L388Y TAT V058G GGT W123Y TAT C189W TGG I256M ATG Y322P CCT E389A GCT V058H CAT K124A GCT C189Y TAT I256N AAT Y322R CGT E389F TTT V058I ATT K124C TGT Y190C TGT I256P CCG Y322S

TCT E389G GGT V058K AAG K124D GAT Y190E GAG I256Q CAG Y322T ACT E389H CAT V058L CTT K124E GAG Y190F TTT I256R AGG Y322V GTG E389I ATT V058N AAT K124F TTT Y190G GGG I256T ACG Y322W TGG E389K AAG V058P CCT K124G GGG Y190H CAT I256V GTT M323A GCT E389L CTG V058Q CAG K124H CAT Y190K AAG I256W TGG M323C TGT E389M ATG V058R CGG K124I ATT Y190L CTT P257A GCG M323E GAG E389P CCT V058S TCG K124L CTT Y190N AAT P257C TGT M323F TTT E389Q CAG V058W TGG K124N AAT Y190P CCT P257D GAT M323G GGG E389R CGG V058Y TAT K124P CCT Y190Q CAG P257G GGG M323H CAT E389S TCG D059A GCT K124R CGG Y190R CGT P257I ATT M323I ATT E389T ACT D059E GAG K124S TCT Y190S TCT P257K AAG M323K AAG E389V GTT D059G GGG K124T ACT Y190T ACT P257L CTT M323L TTG E389Y TAT D059H CAT K124V GTG Y190V GTG P257M ATG M323N AAT D390A GCG D059I ATT K124W TGG Y190W TGG P257N AAT M323P CCT D390C TGT D059L CTT P125A GCT N191A GCT P257Q CAG M323R CGG D390E GAG D059M ATG P125C TGT N191E GAG P257R CGT M323S AGT D390F TTT D059N AAT P125D GAT N191F TTT P257S TCG M323T ACT D390G GGG D059P CCT P125G GGG N191G GGG P257T ACG M323V GTT D390H CAT D059Q CAG P125H CAT N191K AAG P257V GTG E324A GCT D390L CTT D059R CGT P125I ATT N191L TTG P257W TGG E324C TGT D390N AAT D059T ACG P125L CTT N191M ATG D258A GCG E324D GAT D390P CCG D059V GTG P125N AAT N191P CCT D258E GAG E324F TTT D390R CGG D059W TGG P125Q CAG N191Q CAG D258G GGG E324G GGG D390S AGT D059Y TAT P125R CGT N191R CGG D258H CAT E324H CAT D390T ACT R060A GCG P125S TCG N191S TCG D258I ATT E324L TTG D390V GTG R060D GAT P125T ACT N191T ACT D258L CTT E324M ATG D390W TGG R060F TTT P125V GTG N191V GTT D258N AAT E324N AAT D390Y TAT R060G GGT P125W TGG N191W TGG D258P CCG E324P CCT L391A GCT R060H CAT P125Y TAT N191Y TAT D258Q CAG E324R CGG L391C TGT R060I ATT K126A GCT H192C TGT D258R CGT E324S AGT L391D GAT R060K AAG K126D GAT H192F TTT D258S AGT E324V GTG L391G GGG R060L CTT K126E GAG H192G GGT D258T ACG E324W TGG L391H CAT R060N AAT K126F TTT H192K AAG D258V GTG E324Y TAT L391K AAG R060P CCG K126G GGT H192L CTT D258W TGG T325A GCT L391N AAT R060Q CAG K126H CAT H192M ATG D258Y TAT T325C TGT L391P CCT R060S TCG K126I ATT H192N AAT A259E GAG T325D GAT L391Q CAG R060T ACG K126L CTG H192P CCT A259G GGG T325E GAG L391R CGG R060V GTT K126M ATG H192Q CAG A259I ATT T325G GGT L391S TCT R060Y TAT K126N AAT H192R CGT A259K AAG T325H CAT L391T ACT L061A GCT K126P CCT H192S TCG A259L TTG T325I ATT L391V GTG L061E GAG K126Q CAG H192T ACT A259M ATG T325K AAG L391W TGG L061F TTT K126R AGG H192V GTT A259N AAT T325M ATG L391Y TAT L061G GGG K126S TCT H192W TGG A259P CCT T325N AAT E392A GCT L061H CAT K126T ACT H192Y TAT A259Q CAG T325Q CAG E392C TGT L061I ATT K126V GTG H193A GCT A259R CGT T325R CGG E392F TTT L061M ATG K126W TGG H193C TGT A259S AGT T325S TCG E392G GGG L061N AAT K126Y TAT H193D GAT A259T ACT T325V GTG E392K AAG L061P CCT D127A GCT H193F TTT A259V GTG T325W TGG E392L CTG L061Q CAG D127E GAG H193G GGG A259W TGG I326A GCT E392M ATG L061R AGG D127F TTT H193K AAG A259Y TAT I326C TGT E392P CCT L061T ACT D127G GGT H193L TTG K260A GCG I326D GAT E392Q CAG L061V GTT D127H CAT H193M ATG K260C TGT I326E GAG E392R AGG L061W TGG D127K AAG H193P CCG K260D GAT I326G GGG E392S AGT L061Y TAT D127L CTG H193Q CAG

K260E GAG I326H CAT E392T ACT G062A GCG D127M ATG H193R AGG K260G GGG I326K AAG E392V GTT G062C TGT D127N AAT H193S TCT K260H CAT I326L CTT E392W TGG G062D GAT D127Q CAG H193T ACG K260L TTG I326N AAT E392Y TAT G062F TTT D127R CGT H193V GTG K260M ATG I326P CCT Q393A GCG G062I ATT D127S AGT H193Y TAT K260P CCG I326R CGG Q393C TGT G062K AAG D127T ACT Y194A GCT K260Q CAG I326S TCT Q393D GAT G062L CTT D127V GTT Y194C TGT K260R CGG I326V GTG Q393F TTT G062M ATG D127W TGG Y194E GAG K260S TCT I326W TGG Q393G GGT G062P CCT V128A GCT Y194F TTT K260V GTT I326Y TAT Q393H CAT G062Q CAG V128C TGT Y194G GGG K260W TGG L327A GCT Q393I ATT G062R CGT V128E GAG Y194I ATT K260Y TAT L327D GAT Q393K AAG G062S AGT V128F TTT Y194L TTG S261A GCG L327E GAG Q393L TTG G062T ACT V128G GGG Y194N AAT S261E GAG L327F TTT Q393M ATG G062V GTG V128H CAT Y194P CCT S261F TTT L327G GGG Q393N AAT G062Y TAT V128I ATT Y194Q CAG S261G GGG L327H CAT Q393P CCG Y063A GCG V128K AAG Y194R AGG S261I ATT L327M ATG Q393R CGT Y063C TGT V128L CTG Y194S TCG S261K AAG L327N AAT Q393S TCG Y063G GGT V128P CCT Y194T ACG S261L CTT L327Q CAG Q393T ACG Y063H CAT V128Q CAG Y194V GTG S261M ATG L327R CGG F394A GCG Y063I ATT V128R AGG Y194W TGG S261N AAT L327S AGT F394D GAT Y063K AAG V128S TCG K195A GCG S261P CCT L327T ACT F394E GAG Y063L CTG V128W TGG K195E GAG S261Q CAG L327V GTG F394G GGG Y063M ATG V128Y TAT K195F TTT S261R CGT L327W TGG F394I ATT Y063N AAT Y129A GCT K195G GGT S261T ACT L327Y TAT F394K AAG Y063P CCT Y129C TGT K195H CAT S261V GTT N328A GCT F394L CTG Y063R AGG Y129D GAT K195I ATT S261W TGG N328C TGT F394N AAT Y063S TCT Y129E GAG K195L TTG P262A GCG N328D GAT F394P CCG Y063T ACG Y129G GGG K195N AAT P262D GAT N328G GGT F394Q CAG Y063V GTG Y129H CAT K195Q CAG P262E GAG N328H CAT F394R CGT Y063W TGG Y129L TTG K195R CGT P262F TTT N328I ATT F394S TCG Y064A GCT Y129M ATG K195S TCT P262G GGG N328K AAG F394T ACT Y064C TGT Y129P CCT K195T ACT P262H CAT N328L CTT F394V GTT Y064D GAT Y129Q CAG K195V GTG P262I ATT N328Q CAG F394W TGG Y064E GAG Y129R CGG K195W TGG P262K AAG N328R AGG S395A GCG Y064F TTT Y129S AGT K195Y TAT P262Q CAG N328S AGT S395C TGT Y064G GGT Y129T ACT K196A GCT P262R CGT N328T ACT S395D GAT Y064H CAT Y129V GTT K196C TGT P262S TCT N328V GTG S395E GAG Y064I ATT Y129W TGG K196D GAT P262T ACT N328W TGG S395G GGG Y064K AAG K130C TGT K196E GAG P262V GTG N328Y TAT S395H CAT Y064L CTT K130D GAT K196G GGG P262W TGG P329C TGT S395K AAG Y064P CCT K130E GAG K196I ATT P262Y TAT P329F TTT S395L CTT Y064Q CAG K130G GGG K196L TTG L263A GCT P329G GGT S395M ATG Y064R CGG K130H CAT K196N AAT L263E GAG P329H CAT S395P CCT Y064S AGT K130I ATT K196P CCG L263F TTT P329I ATT S395R CGG Y064T ACT K130L TTG K196R CGT L263G GGG P329K AAG S395T ACG Y064V GTT K130N AAT K196S TCG L263H CAT P329L CTG S395V GTT Y064W TGG K130Q CAG K196T ACT L263K AAG P329N AAT S395W TGG P065A GCT K130R AGG K196V GTG L263M ATG P329Q CAG S395Y TAT P065C TGT K130S TCT K196W TGG L263N AAT P329R CGT E396A GCG P065D GAT K130T ACT K196Y TAT L263P CCG P329S AGT E396C TGT P065F TTT K130V GTG P197A GCT L263Q CAG P329T ACT E396D GAT P065G GGG K130W TGG P197C TGT L263R CGG P329V GTT E396F TTT P065H CAT K130Y TAT P197D GAT L263S AGT P329W TGG E396G GGG P065I ATT N131C TGT

P197E GAG L263T ACT P329Y TAT E396H CAT P065K AAG N131E GAG P197F TTT L263V GTT Y330A GCT E396I ATT P065N AAT N131F TTT P197G GGT L263W TGG Y330C TGT E396L CTT P065R CGG N131G GGG P197H CAT P264A GCG Y330D GAT E396P CCG P065S TCG N131H CAT P197K AAG P264D GAT Y330E GAG E396Q CAG P065T ACG N131I ATT P197L TTG P264E GAG Y330F TTT E396R AGG P065V GTT N131L CTT P197M ATG P264F TTT Y330G GGT E396S TCT P065W TGG N131M ATG P197Q CAG P264G GGT Y330I ATT E396T ACT P065Y TAT N131P CCT P197R CGT P264H CAT Y330L CTG E396V GTG Y066A GCG N131Q CAG P197S AGT P264L CTT Y330M ATG E396Y TAT Y066C TGT N131R CGG P197T ACT P264M ATG Y330N AAT K397A GCT Y066D GAT N131S AGT P197W TGG P264N AAT Y330P CCT K397C TGT Y066E GAG N131T ACT G198A GCT P264R CGG Y330R AGG K397E GAG Y066G GGT N131V GTG G198C TGT P264S AGT Y330S AGT K397F TTT Y066H CAT N131Y TAT G198D GAT P264T ACT Y330V GTT K397G GGT Y066I ATT R132A GCT G198E GAG P264V GTT I331V GTG K397I ATT Y066K AAG R132C TGT G198H CAT P264W TGG Y330W TGG K397L TTG Y066L CTG R132E GAG G198L CTG P264Y TAT I331A GCT K397M ATG Y066N AAT R132F TTT G198N AAT V265A GCG I331C TGT K397N AAT Y066P CCT R132H CAT G198P CCG V265C TGT I331D GAT K397P CCG Y066R CGG R132I ATT G198Q CAG V265D GAT I331E GAG K397Q CAG K397T ACT R132K AAG G198R AGG V265E GAG I331F TTT K397R AGG K397V GTT R132L TTG G198S TCT V265F TTT I331H CAT K397S TCG F398A GCT L406P CCT K415G GGT C423T ACT A432L TTG E441D GAT F398C TGT L406Q CAG K415L CTG C423V GTG A432M ATG E441F TTT F398E GAG L406R CGG K415M ATG C423W TGG A432N AAT E441G GGG F398G GGT L406S AGT K415P CCG I424A GCT A432P CCT E441H CAT F398H CAT L406T ACG K415Q CAG I424C TGT A432R AGG E441K AAG F398I ATT L406V GTT K415R CGG I424E GAG A432S TCT E441L CTT F398L CTT L406Y TAT K415S TCT I424G GGG A432V GTG E441N AAT F398N AAT S407A GCG K415T ACT I424H CAT A432Y TAT E441Q CAG F398P CCT S407D GAT K415V GTG I424K AAG F433A GCT E441R CGG F398R AGG S407E GAG K415W TGG I424L CTT F433C TGT E441S AGT F398S TCT S407F TTT K415Y TAT I424N AAT F433D GAT E441T ACT F398T ACT S407G GGT D416C TGT I424Q CAG F433E GAG E441V GTG F398V GTT S407H CAT D416F TTT I424R CGG F433G GGG E441Y TAT F398W TGG S407L CTG D416G GGT I424S TCG F433H CAT E442C TGT F398Y TAT S407M ATG D416H CAT I424T ACT F433I ATT E442G GGG Y399A GCG S407N AAT D416I ATT I424V GTT F433K AAG E442H CAT Y399C TGT S407P CCT D416K AAG I424W TGG F433L TTG E442K AAG Y399D GAT S407Q CAG D416L CTT I424Y TAT F433P CCT E442L CTT Y399E GAG S407R CGG D416N AAT A425C TGT F433R CGG E442M ATG Y399G GGG S407T ACG D416Q CAG A425D GAT F433S AGT E442N AAT Y399K AAG S407V GTG D416R CGG A425E GAG F433T ACT E442P CCT Y399M ATG S407W TGG D416S TCT A425G GGT F433V GTG E442Q CAG Y399N AAT C408A GCG D416T ACG A425I ATT F433W TGG E442R CGG Y399P CCT C408E GAG D416V GTG A425K AAG L434F TTT E442S AGT Y399Q CAG C408F TTT D416W TGG A425L TTG L434G GGT E442T ACT Y399R CGG C408G GGG D416Y TAT A425M ATG L434H CAT E442V GTG Y399S TCG C408I ATT T417A GCT A425N AAT L434I ATT E442W TGG Y399T ACG C408K AAG T417D GAT A425P CCT L434K AAG E442Y TAT Y399V GTT C408L CTT T417E GAG A425R AGG L434M ATG P443A GCT Y399W TGG C408N AAT T417F TTT A425S AGT L434N AAT P443D GAT C400A GCG C408P CCT T417G GGG A425V GTG L434P CCT P443E GAG C400D GAT

C408R CGT T417H CAT A425W TGG L434Q CAG P443F TTT C400E GAG C408S TCG T417I ATT A425Y TAT L434R CGG P443G GGG C400F TTT C408T ACT T417K AAG D426A GCT L434S AGT P443H CAT C400G GGG C408V GTT T417L TTG D426C TGT L434T ACT P443I ATT C400I ATT C408W TGG T417M ATG D426E GAG L434V GTT P443L CTT C400L CTG C408Y TAT T417P CCT D426F TTT L434W TGG P443M ATG C400M ATG K409A GCG T417Q CAG D426G GGG L434Y TAT P443N AAT C400P CCG K409C TGT T417R CGT D426I ATT K435A GCT P443Q CAG C400Q CAG K409D GAT T417S TCG D426K AAG K435C TGT P443R AGG C400R CGG K409E GAG T417W TGG D426L CTG K435E GAG P443S TCT C400S AGT K409G GGT D418A GCT D426M ATG K435F TTT P443T ACT C400T ACG K409H CAT D418C TGT D426N AAT K435G GGT P443W TGG C400V GTG K409I ATT D418E GAG D426P CCT K435H CAT Q444C TGT C400Y TAT K409L CTG D418F TTT D426Q CAG K435I ATT Q444D GAT S401A GCT K409P CCG D418G GGT D426R CGT K435L CTG Q444E GAG S401C TGT K409Q CAG D418I ATT D426S TCG K435P CCT Q444F TTT S401D GAT K409R AGG D418L TTG D426Y TAT K435R AGG Q444G GGG S401E GAG K409S TCG D418M ATG G427A GCT K435S TCT Q444H CAT S401F TTT K409T ACG D418N AAT G427C TGT K435T ACT Q444I ATT S401G GGG K409V GTG D418P CCT G427F TTT K435V GTT Q444K AAG S401H CAT K409W TGG D418Q CAG G427H CAT K435W TGG Q444L CTG S401K AAG A412Y TAT D418R CGG G427I ATT K435Y TAT Q444M ATG S401L CTT E410D GAT D418S TCG G427K AAG P436C TGT Q444N AAT S401N AAT E410G GGG D418V GTG G427L CTG P436D GAT Q444R CGG S401Q CAG E410I ATT D418Y TAT G427P CCT P436E GAG Q444V GTT S401R CGT E410K AAG A419D GAT G427Q CAG P436G GGG Q444W TGG S401T ACT E410L CTT A419E GAG G427R CGT P436H CAT Q444Y TAT S401W TGG E410M ATG A419F TTT G427S AGT P436I ATT I445A GCT S401Y TAT E410N AAT A419G GGG G427T ACT P436K AAG I445C TGT C402A GCT E410P CCG A419H CAT G427V GTG P436L CTG I445D GAT C402D GAT E410Q CAG A419I ATT G427W TGG P436M ATG I445G GGG C402E GAG E410R CGT A419K AAG G427Y TAT P436Q CAG I445H CAT C402F TTT E410S TCG A419L CTT V428A GCT P436R CGG I445K AAG C402G GGG E410T ACG A419N AAT V428C TGT P436S TCT I445L CTT C402L TTG E410V GTG A419P CCT V428D GAT P436T ACT I445M ATG C402M ATG E410W TGG A419R CGG V428E GAG P436W TGG I445N AAT C402P CCT E410Y TAT A419S TCT V428F TTT P436Y TAT I445P CCT C402Q CAG K411A GCT A419T ACT V428G GGT P437A GCT I445Q CAG C402R CGG K411D GAT A419W TGG V428H CAT P437D GAT I445R AGG C402S TCT K411E GAG A419Y TAT V428L CTT P437F TTT I445S AGT C402T ACG K411F TTT V420A GCT V428M ATG P437G GGT I445T ACT C402V GTT K411G GGG V420D GAT V428N AAT P437H CAT I445V GTG C402W TGG K411H CAT V420F TTT V428P CCT P437I ATT I445W TGG C402Y TAT K411I ATT V420G GGT V428R CGG P437K AAG I445Y TAT Y403A GCT K411L CTG V420H CAT V428S TCG P437L CTG F446A GCT Y403C TGT K411N AAT V420I ATT V428T ACT P437M ATG F446C TGT Y403E GAG K411P CCT V420K AAG V428Y TAT P437Q CAG F446D GAT Y403F TTT K411R AGG V420L CTT C429A GCT P437R CGT F446E GAG Y403G GGT K411S TCG V420N AAT C429D GAT P437S TCT F446G GGG Y403H CAT K411T ACT V420P CCT C429G GGT P437T ACT F446H CAT Y403K AAG K411V GTT V420R AGG C429I ATT P437W TGG F446I ATT Y403L TTG K411W TGG V420S TCT C429K AAG P437Y TAT F446K AAG Y403M ATG A412D GAT V420T ACT C429L TTG M438A GCT F446L TTG Y403N AAT A412E GAG V420W TGG C429M ATG M438C TGT

F446M ATG Y403P CCG A412G GGG V420Y TAT C429N AAT M438D GAT F446Q CAG Y403Q CAG A412H CAT D421A GCT C429P CCT M438E GAG F446R CGG Y403R CGG A412I ATT D421E GAG C429R CGG M438G GGG F446T ACT Y403S TCT A412L CTG D421G GGT C429S TCG ~~M438L~~M348L TTG F446V GTT Y403T ACG A412N AAT D421H CAT C429T ACT M438N AAT F446W TGG S404A GCT A412P CCT D421I ATT C429V GTT M438P CCT Y447D GAT S404C TGT A412Q CAG D421K AAG C429W TGG M438Q CAG Y447E GAG S404D GAT A412R CGG D421L TTG C429Y TAT M438R AGG Y447F TTT S404F TTT A412S AGT D421M ATG I430A GCT M438S TCG Y447G GGT S404G GGT A412V GTT D421N AAT I430D GAT M438T ACT Y447I ATT S404H CAT A412W TGG D421Q CAG I430E GAG M438V GTG Y447K AAG S404L CTT D413A GCG D421R CGG I430G GGG M438W TGG Y447L CTT S404M ATG D413E GAG D421S TCG I430H CAT M438Y TAT Y447M ATG S404N AAT D413F TTT D421T ACT I430K AAG E439A GCT Y447N AAT S404P CCT D413G GGT D421W TGG I430L TTG E439C TGT Y447P CCT S404R AGG D413H CAT D421Y TAT I430M ATG E439F TTT Y447Q CAG S404T ACG D413I ATT V422A GCT I430N AAT E439G GGG Y447R AGG S404V GTG D413K AAG V422C TGT I430P CCT E439H CAT Y447T ACT S404W TGG D413L CTG V422D GAT I430R AGG E439K AAG Y447V GTT S404Y TAT D413N AAT V422E GAG I430S TCT E439L CTT Y447W TGG T405A GCG D413P CCG V422G GGG I430T ACT E439N AAT T405C TGT D413Q CAG V422H CAT I430V GTT E439P CCT T405F TTT D413R CGT V422I ATT I430W TGG E439Q CAG T405G GGG D413S TCG V422L CTG D431A GCT E439R CGG T405I ATT D413T ACT V422M ATG D431E GAG E439S TCG T405K AAG D413W TGG V422N AAT D431G GGT E439T ACT T405L TTG V414A GCG V422P CCT D431H CAT E439V GTT T405M ATG V414D GAT V422Q CAG D431I ATT E439W TGG T405P CCG V414E GAG V422R CGT D431K AAG T440A GCT T405Q CAG V414F TTT V422S TCG D431L CTT T440D GAT T405R CGT V414G GGT V422T ACT D431N AAT T440E GAG T405S TCT V414H CAT V422W TGG D431P CCT T440F TTT T405V GTG V414I ATT V422Y TAT D431Q CAG T440G GGG T405W TGG V414K AAG C423A GCT D431R CGT T440H CAT T405Y TAT V414L TTG C423D GAT D431S TCT T440I ATT L406A GCT V414M ATG C423E GAG D431V GTT T440L CTT L406C TGT V414Q CAG C423F TTT D431W TGG T440M ATG L406D GAT V414R AGG C423G GGG D431Y TAT T440P CCT L406E GAG V414S TCG C423H CAT A432C TGT T440Q CAG L406F TTT V414T ACT C423L CTG A432E GAG T440R AGG L406G GGT V414Y TAT C423M ATG A432F TTT T440S AGT L406I ATT K415A GCG C423P CCT A432G GGG T440V GTG L406N AAT K415C TGT C423Q CAG A432H CAT T440Y TAT K415D GAT C423R AGG A432I ATT E441A GCT K415E GAG C423S TCG A432K AAG E441C TGT

~~[0607]~~(509) 2. Expression

~~[0608]~~(510) For expression of each mutant, HZ24-PH20-IRES-SEAP plasmid DNA containing cDNA encoding one of the variant PH20 or encoding wildtype PH20 was transfected into monolayer ~~CHO-S~~CHO—S cells (Invitrogen, Cat. No. 11619-012) using Lipofectamine 2000 (Invitrogen, Cat. No. 11668-027) according to the protocol suggested by the manufacturer. ~~CHO-S~~CHO—S cells were seeded the night before transfection and grown in DMEM with 10% FBS to be 80% confluent the next day. Then, the medium of the ~~CHO-S~~CHO—S cells was replaced with Opti-MEM. A mixture of plasmid DNA and lipofectamine was made (0.2 ~~.mu.g~~µg DNA and 0.5 ~~.mu.L Lipofetamine).~~µL Lipofectamine) The Lipofectamine/DNA mixture was added to ~~CHO-S~~CHO—S cells and incubated overnight. The next day, the cells were supplemented with

CD-CHO serum free medium (Invitrogen, Cat. No. 10743-029). Supernatant from transfected cells was collected at various time points after transfection, and generally 96 hours after transfection. The supernatant, containing the variant PH20 protein or wildtype PH20 having a sequence of amino acids set forth in SEQ ID NO:3, was stored at ~~-20.degree.~~−20° C. Activities of the supernatants were screened as described in the following examples.

Example 3

Screening of Library with a Hyaluronidase Activity Assay to Identify Activity Mutants

~~[0609]~~(511) In this example, supernatants of expressed PH20 variants generated in Example 2 were screened using a hyaluronidase activity assay to assess activity of each mutant. In addition, activity of the secreted alkaline phosphatase (SEAP) was also measured to allow for normalizing PH20 activity of the expressed mutants to the PH20 wildtype. Active and inactive mutants were identified.

~~[0610]~~(512) 1. Generation of Biotinylated HA (bHA) Substrate

~~[0611]~~(513) A 1.2-MDa ~~FLA~~HA (Lifecore) was biotinylated for use as a substrate in the hyaluronidase activity assay. First, 1.2 grams (g) of 1.2 MDa HA was ~~dissovled~~dissolved at 4~~.degree.~~° C. in 600 mL ddH.sub.2O for a week at a concentration of 2 mg/mL with stirring. Next, 645.71 mg Biotin Hydrazide was dissolved in 100 mL DMSO to a concentration of 25 mM (6.458 mg/mL, 247.8 mg in 38.37 mL DMSO). The biotin solution was warmed briefly at 37~~.degree.~~° C. until the solution was clear. Also, 368.61 mg Sulfo-NHS in 20 mL ddH.sub.2O was dissolved to make a 100~~.times.~~× solution (18.4 mg/mL Sulfo-NHS). A 30 mM (1000~~.times.~~×) water-soluble carbodiimide EDC solution was made by dissolving 17.63 mg EDC in 3 mL ddH20 at a concentration of 5.7513 mg/mL right before the reaction was started.

~~[0612]~~(514) To four (4) 1000-mL sterile capped bottles, the following components were added at room temperature (RT) and in the following order with stirring: 1) 200 mL of 2 mg/mL HA solution; 2) 80 mL of 0.5M MES, pH 5.0 with gentle mixing; and 3) 91.6 mL of ddH.sub.2O with gentle mixing. Next, 24 mL of 25 mM Biotin-Hydrazide and 4 mL of 100~~.times.~~× Sulfo-NHS solution were added sequentially, immediately followed by the addition of 500 ~~.mu.L~~µL EDC. After the addition of each component, the solution was mixed by inverting three times and stirring. After the addition of the last component, the solution was mixed by stirring overnight at 4~~.degree.~~° C. Then, Guanidine hydrochloride was added to a final concentration of 4 M by adding 38.2 g per 100 mL and was allowed to dissolve completely before adjusting the solution volume to 600 mL with ddH.sub.2O.

~~[0613]~~(515) For dialysis, 200 mL from each batch of the conjugated HA guanidine hydrochloride solution was transferred into dialysis membranes. Over the course of three days, the solution was dialyzed against ddH.sub.2O with a change in ddH.sub.2O at least six times. The resulting volume of about 840 mL was adjusted to a final volume of 1000 mL with ddH.sub.2O. The final concentration of the biotinylated hyaluronan (bHA) was 0.4 mg/mL.

~~[0614]~~(516) 2. Hyaluronidase Activity Assay

[0615](517) The enzyme assay was a modification of the method described by Frost et al. (1997) (A Microtiter-Based Assay for Hyaluronidase Activity Not Requiring Specialized Reagents. Analytical Biochemistry (1997) 251:263-269) that provides a measure of PH20 hyaluronidase activity.

[0616](518) First, biotinylated HA (bHA) substrate was bound to plastic microtiter plates to generate assay plates. Briefly, 100 .mu.lµl of b-HA at 1 mg/mL in 0.5 M carbonate buffer (pH 9.6) was dispensed into each well of a high bind microplate (Immunolon 4 HBX extra high binding; Thermo Scientific). The plate was covered with a plate sealer and stored between 2-8.degree.° C. for 24-48 hours.

[0617](519) Then, the assay plate was washed with 1.times.× phosphate buffered saline (PBS) wash buffer containing 0.05% (v/v) Tween 20 (PBST). PBST was generated from 1.times.×PBS (generated from Catalog No. P5368, Sigma (10 mM Phosphate Buffer, 2.7 mM Potassium Chloride, 137 mM Sodium Chloride, pH 7.4) by placing the contents of one packet of PBS into a 1-L graduated cylinder with 800 mL deionized water, dissolved by stirring or shaking and adding sufficient quantity of water to 1 L) by adding 500 .mu.lµl Tween 20 (Catalog No. 6505; EMD Bioscience) to 900 mL of 1.times.×PBS and adding sufficient quantity of water to 1 L. Washing was done using the BioTek ELx405 Select CW plate washer (BioTek) by washing five (5) times with 300 .mu.lµl PBST wash buffer per well for each wash. At the end of each wash, the plate was tapped on a paper towel to remove excess liquid from each well. Prior to incubation with samples, 200 .mu.lµl Blocking Buffer (1.0% w/v Bovine Serum Albumin (BSA) in PBS) was added to each well and the assay plate was incubated at 37.degree.° C. for approximately 1 hour prior. The Blocking buffer was generated by adding 2.5 g of BSA (Catalog No. 001-000-162; Jackson Immuno Research) to 200 mL 1.times.×PBS, stirring, adding a sufficient quantity of 1.times.×PBS to 250 mL and filtering through an 0.2 .mu.MµM PES filter unit.

[0618](520) Transfected variant or wildtype PH20 supernatants generated as described in Example 1 were diluted in duplicate 1:25 in assay diluent buffer (pH 7.4 HEPES buffer; 10 mM HEPES, 50 mM NaCl, 1 mM CaCl.sub.2, 1 mg/mL BSA, pH 7.4.degree., 0.05% Tween-20) in uncoated 4.times.HB4XHB high bound microplates. For the standard curve, 1:3 serial dilutions of rHuPH20 (generated as described in Example 1 with a specific activity of 145 U/mL) were made in assay diluent buffer in duplicate starting from 3 U/mL for standards as follows: 3 U/mL, 1 U/mL, 1/3⅓ U/mL, 1/9 U/mL, 1/27 U/mL, 1/81 U/mL, and 1/243 U/mL. One hundred microliters (100 .mu.lµl) of each standard and sample were transferred to the assay plates and incubated for approximately 1.5 hours at 37.degree.° C.

[0619](521) After the incubation, the plate was washed with PBST using the BioTek ELx405 Select CW plate washer by washing five (5) times with 300 .mu.lµl PBST wash buffer per well for each wash. At the end of each wash, the plate was tapped on a paper towel to remove excess liquid from each well. Then, 100 .mu.lµl of 1:5000 diluted Streptavidin-HRP (SA-HRP) was added to each well of the plate and incubated at ambient temperature for approximately 1 hour. For the dilution, a 1 mg/mL stock of Streptavidin-HRP conjugate (Catalog No. 21126; Thermo Scientific) was diluted 1:5000 into dilution buffer (1 mg/mL BSA, 0.025% Tween20, 137 mM NaCl, 20 mM Tris pH 7.5). After the incubation, the plate was washed with PBST using the BioTek ELx405 Select CW plate washer by washing five (5) times with 300 .mu.lµl PBST wash buffer per well for each wash. At the end of each wash, the plate was tapped on a paper towel to

remove excess liquid from each well. Then, 100 ~~.mu.l~~µl of TMB solution (Catalog No. 52-00-03, KPL; ambient temperature and protected from light) was added to each well for approximately five (5) minutes at room temperature or until an optimal color development was yielded. To stop the reaction, 100 ~~.mu.l~~µl 1.0 N Sulfuric Acid or TMB Stop solution (Catalog No. 50-85-06) were added to each well and the plates tapped to mix. Optical density was measured at 450 nm within 30 minutes of adding the stop solution. Since more PH20 in a standard or sample would lead to less bHA available to bind SA-HRP, the optical density (450 nm) value was inversely proportional to the concentration of hyaluronidase activity in each specimen.

~~[0620]~~(522) 3. SEAP Activity

~~[0621]~~(523) Activity of secreted alkaline phosphatase (SEAP) in the cell culture supernatant also was measured using a colorimetric assay of placental alkaline phosphatase using pNPP as a phosphatase substrate (Anaspec SensoLyte pNPP SEAP kit; Catalog No. 72144, Anaspec) according to the manufacturer's instructions. The absorbance signal was measured at optical density (OD) of 405 nm.

~~[0622]~~(524) The criteria for the high throughput (HTP) screening were that the transfected supernatant resulted in a SEAP signal of ~~.gtoreq.~~≥0.1 and the signal for the rHuPH20 wildtype control produced a signal of ~~.gtoreq.~~≥1 U/mL. Also, the criteria for each screen were that the standard curves had a signal to noise ratio (S/N) for the 0 U/mL standard versus the 3 u/mL standard at OD.sub.405 of ~~.gtoreq.~~≥5, had less than three (3) standards with a coefficient of variation (CV) ~~.gtoreq.~~≥10%, and at least four (4) of the standards were in the linear range.

Example 4

Selected PH20 Variants with Altered Hyaluronidase Activity

~~[0623]~~(525) Each generated variant was screened for ~~hyaluonidase~~hyaluronidase activity as described in Example 3. The SEAP expression was used to normalize PH20 activity of each variant to the PH20 wildtype. Mutants were identified that exhibited altered hyaluronidase activity compared to wildtype.

~~[0624]~~(526) 1. Active Mutants

~~[0625]~~(527) Active mutants were selected whereby at least one duplicate sample exhibited greater than 40% of wildtype activity when normalized to SEAP activity. The identified active mutants are set forth in Table 9. The Table sets forth the amino acid replacement compared to the sequence of amino acids of PH20 set forth in SEQ ID NO:3. The amino acid sequence of exemplary mutants also is set forth by reference to a SEQ ID NO. The Table also sets forth the average hyaluronidase activity of tested duplicates normalized by SEAP values compared to average of wildtype PH20 activities in each plate, which were also normalized by their own SEAP values. For example, a value of 0.40 indicates that the variant exhibits 40% of the hyaluronidase activity of wildtype PH20, a value of 1 indicates that the variant exhibits a similar hyaluronidase activity of wildtype and a value of 3.00 indicates that the variant exhibits 300% of the hyaluronidase activity of wildtype PH20 or 3-fold increased activity compared to wildtype.

~~[0626]~~(528) The results in Table 9 show that over 600 tested mutants exhibit activity that is increased compared to wildtype. For example, about 536 mutants exhibit 120% or greater than 120% of the hyaluronidase activity of wildtype PH20 and about 75 of the mutants exhibit 300% or greater than 300% of the hyaluronidase activity of wildtype PH20. In particular, the results in Table 9 show that that hyaluronidase activity compared to wildtype of mutant S69A is about 22-fold; mutant S69R is about 14-fold; mutant ~~I70A~~170A is about 27-fold; mutant ~~I70K~~170K is about 14-fold; mutant ~~I70R~~170R is about 14-fold; and mutant I271L is about 10-fold.

(529) TABLE-US-00009 TABLE 9 ACTIVE MUTANTS SEQ Avg- SEQ Avg- SEQ Avg- ID ~~AvgNorm~~Norm ID Norm ID Norm mutant ~~NO~~No Act. mutant No Act. mutant No Act. L001A 74 0.95 Q140G 0.73 T293F 561 1.94 L001C 0.89 Q140H 0.84 T293G 1.00 L001E 75 0.55 Q140I 0.75 T293K 562 1.35 L001F 0.41 Q140K 343 0.93 T293L 1.00 L001G 76 0.62 Q140L 0.51 T293M 563 2.29 L001H 73 1.90 Q140M 0.80 T293P 564 1.64 L001K 77 1.39 Q140R 0.85 T293Q 565 1.83 L001N 0.87 Q140V 0.61 T293S 0.89 L001P 0.92 Q140W 0.59 T293V 566 2.15 L001Q 78 3.27 Q140Y 0.41 T293Y 567 1.49 L001R 79 0.72 N141A 1.12 V294M 0.41 L001S 0.74 N141D 1.09 A298G 568 0.43 L001T 0.99 N141E 0.67 A298I 0.41 L001V 1.00 N141F 0.81 G300R 0.42 L001W 0.88 N141G 1.15 I301A 0.88 N002A 0.61 N141H 344 2.03 I301V 0.88 N002C 0.4 N002I 0.37 V287N 0.35 G291C 0.27 G297A 0.57 V302W 0.46 N002G 0.44 N141L 0.61 V302I 0.45 N002L 0.46 N141M 0.48 I303V 0.47 N002P 0.54 N141Q 1.16 W304G 1.13 N002Q 0.84 N141R 345 1.40 W304I 1.17 N002S 0.78 N141S 346 0.72 G305D 1.00 N002T 1.05 N141T 0.45 G305E 569 1.62 N002V 0.65 N141V 0.50 T306D 0.76 F003E 0.42 N141W 347 0.83 T306E 0.52 F003H 0.68 N141Y 348 1.55 T306S 1.02 F003L 0.59 V142C 0.61 L307K 0.43 F003Y 0.50 V142D 349 0.71 L307N 0.76 R004A 0.73 V142E 0.87 L307Q 0.61 R004I 0.54 V142G 350 0.98 L307S 0.86 R004S 0.60 V142H 1.11 L307T 1.08 R004T 0.66 V142I 0.81 L307V 0.48 R004V 1.09 V142K 351 1.40 L307W 0.64 A005H 0.44 V142L 0.75 L307Y 0.60 P006A 80 0.78 V142M 0.76 S308D 571 0.92 P006H 0.58 V142N 352 0.98 S308G 572 1.73 P006K 0.80 V142P 353 0.88 S308H 1.15 P006L 0.76 V142Q 354 1.04 S308K 573 1.33 P006N 0.40 V142R 355 1.53 S308N 574 2.33 P006Q 0.89 V142S 356 0.93 S308P 0.65 P006R 0.56 V142T 357 1.19 S308R 575 1.34 P007M 0.57 Q143E 0.77 S308T 0.72 V008I 1.17 Q143G 358 0.62 I309D 0.72 V008L 0.53 Q1431 0.44 I309E 576 1.99 V008M 81 0.47 Q143K 359 1.30 I309G 577 1.44 V008P 0.33 I009Q 82 0.4 I303D 0.34 I009K 0.69 Q143L 0.56 I309H 578 1.30 I009L 1.08 Q143N 0.73 I309K 0.98 I009R 0.53 Q143V 0.57 I309L 579 1.72 I009S 0.98 L144T 361 1.02 I309M 580 1.47 I009V 0.84 L144W 0.79 I309N 581 3.11 P010D 0.62 S145A 0.58 I309Q 582 1.64 P010E 0.66 S145C 0.44 I309R 583 2.27 P010G 83 0.55 S145D 0.48 I309S 584 1.16 P010H 84 0.43 S145E 0.56 I309T 585 2.09 P010N 0.55 S145G 0.94 I309V 586 0.60 P010Q 0.89 S145H 0.56 I309W 0.88 P010R 0.73 S145L 0.44 M310A 587 1.50 P010S 0.55 S145M 0.56 M310G 588 2.73 P010W 0.59 S145N 0.58 M310Q 589 0.59 N011D 0.54 S145P 1.04 M310R 0.50 N011G 0.45 S145R 0.97 M310S 590 1.61 N011H 0.69 L146A 0.52 M310V 0.70 N011K 0.58 L146C 0.42 R311G 0.53 N011S 85 0.39 G305N 0.36 L307G 570 0.32 M310F 0.30 M310Y 0.38 R311G 0.54 V012A 0.56 L146E 0.50 R311H 0.48 V012E 86 1.86 L146G 0.62 R311K 0.72 V012I 87 0.68 L146H 0.78 R311Q 0.43 V012K 88 0.65 L146I 0.82 R311S 0.84 V012L 0.44 L146K 0.84 R311T 0.52 V012N 0.46 L146N 0.57 S312G 0.49 V012R 0.50 L146P 362 0.93 S312N 1.26 V012S 0.75 L146Q 0.84 S312T 0.75 V012T 89 1.50 L146R 363 1.47 M313A 591 1.34 P013H 0.46 L146S 0.71 M313E 0.63 P013S 0.68 L146T 0.74 M313G 592 0.56 P013T 0.90 L146V 0.84 M313H 593 1.23 P013Y 0.51 L146Y 0.80 M313K 594 2.85 F014D 0.64 S312K 0.38 S312L 0.38 F014I 0.42 T147A 364 1.20 M313L 1.05 F014M 0.47 T147C 0.47 M313P 595 1.11 F014V 90 0.46 T147D 0.71 M313R 596 2.30 L015A 0.65 T147F

365 1.24 M313S 0.88 L015M 92 0.45 T147G 1.05 M313T 597 0.67 L015V 91 2.20 T147I 0.85 M313V 0.99 A020S 93 0.50 T147L 366 1.30 M313Y 598 1.12 S022H 0.57 T147M 0.79 K314A 0.82 S022M 0.49 T147P 1.09 K314D 0.53 S022T 94 0.48 T147Q 1.29 K314H 1.10 S022Y 0.45 T147R 367 2.11 K314I 0.54 E023D 0.97 T147S 368 1.27 K314N 0.57 F024A 0.69 T147V 369 2.04 K314Q 0.62 F024E 95 3.99 T147W 0.97 K314R 0.95 F024G 0.75 T147Y 1.04 K314S 599 0.61 F024H 96 2.07 E148C 0.66 K314T 0.61 F024I 0.70 E148F 0.42 K314Y 600 0.45 F024K 0.96 E148G 1.05 S315A 601 0.85 F024L 0.62 E148H 370 1.24 S315E 0.41 F024M 0.85 E148I 0.73 S315G 0.72 F024N 0.60 E148K 371 1.63 S315H 602 2.04 F024R 97 1.22 E148L 0.85 S315K 0.62 F024T 1.18 E148Q 372 1.44 S315L 0.42 F024V 1.15 E148R 0.97 S315M 0.63 F024Y 0.90 E148S 1.15 S315R 1.04 L026A 98 1.30 E148T 0.82 S315T 0.97 L026E 99 3.22 E148V 0.99 S315Y 603 0.50 L026G 0.81 E148W 0.43 C316D 0.41 L026H 0.97 E148Y 0.95 L317A 604 1.27 L026I 0.51 A149C 1.15 L317D 0.61 L026K 100 1.88 A149G 0.52 L317H 1.05 L026M 101 1.43 A149K 0.51 L317I 605 1.76 L026P 0.55 A149L 0.88 L317K 606 5.11 L026Q 102 1.44 A149M 0.88 L317M 1.20 L026R 103 1.43 A149Q 1.15 L317N 607 0.73 L026S 0.78 A149R 1.02 L317Q 608 1.67 L026T 0.87 A149S 1.08 L317R 609 2.41 L026V 0.52 A149T 373 1.24 L317S 610 1.03 L026W 0.53 A149V 374 1.34 L317T 611 0.93 L026Y 0.52 T150A 375 1.21 L317W 612 0.84 G027A 0.79 T150C 0.70 L318D 614 0.46 G027D 104 1.22 T150D 376 1.24 L318F 0.51 G027E 1.18 T150E 1.05 L318G 0.49 G027F 0.61 T150F 0.71 L318H 615 0.45 G027H 1.11 T150G 377 2.19 L318I 0.70 G027I 0.41 T150I 0.52 L318K 616 1.36 G027K 105 2.71 T150L 0.70 L318M 613 1.68 G027L 0.76 T150N 378 0.91 L318N 0.52 G027P 0.46 T150P 0.88 L318Q 0.71 G027Q 1.12 T150R 0.90 L318R 617 1.34 G027R 106 1.88 T150S 379 0.92 L318S 0.71 G027S 0.94 T150W 380 1.25 L318T 0.63 G027T 0.61 T150Y 381 1.36 D320E 0.78 G027W 0.76 E151A 382 1.27 D320G 0.83 K028A 0.78 E151C 1.00 D320H 618 1.75 K028D 0.62 E151G 1.06 D320I 1.00 K028E 0.54 E151H 383 1.34 D320K 619 6.42 K028F 0.75 E151K 384 2.05 D320M 0.79 K028I 0.55 E151L 385 1.03 D320N 0.52 K028L 0.51 E151M 386 1.26 D320R 620 3.19 K028M 0.67 E151N 0.95 D320S 1.19 K028N 0.58 E151Q 387 2.01 D320W 0.40 K028P 0.40 D320L 0.37 D320V 0.35 K028R 107 0.71 E151R 388 1.61 D320Y 0.86 K028S 0.46 E151S 389 1.28 N321A 1.01 K028T 0.68 E151T 390 1.21 N321D 1.25 K028V 0.76 E151V 391 1.38 N321H 0.92 K028W 0.51 E151W 392 1.31 N321K 1.29 F029A 0.90 E151Y 393 1.31 N321R 621 1.23 F029E 108 4.03 K152A 0.51 N321S 622 1.26 F029G 1.05 K152C 0.52 N321T 0.64 F029H 0.82 K152F 0.61 N321Y 0.40 F029I 109 1.53 K152I 0.65 M323F 0.64 F029K 110 1.34 K152M 0.75 M323I 0.55 F029L 111 2.36 K152R 394 1.85 M323L 0.55 F029M 112 2.08 K152T 395 1.20 E324A 0.59 F029P 113 3.79 K152V 0.82 E324D 1.15 F029R 114 1.24 K152Y 0.67 E324H 0.79 F029S 115 2.21 A153I 0.93 E324M 0.50 F029T 116 0.85 A153L 0.51 E324N 623 1.01 F029V 117 1.65 K154R 0.86 E324R 624 2.28 F029W 0.48 K154T 0.83 E324S 0.62 D030A 1.12 K154V 0.46 T325A 625 1.87 D030F 0.84 Q155A 0.91 T325D 626 1.78 D030G 118 2.02 Q155C 0.60 T325E 627 4.03 D030H 119 1.69 Q155D 397 1.49 T325G 628 4.21 D030K 120 2.63 Q155F 0.70 T325H 629 3.45 D030L 121 1.32 Q155G 398 1.61 T325K 630 4.37 D030M 122 1.85 Q155H 1.03 T325M 631 2.11 D030P 1.19 Q155K 399 1.57 T325N 632 4.64 D030Q 0.84 Q155L 0.86 T325Q 633 5.08 D030R 123 1.82 Q155M 0.97 T325S 634 3.19 D030S 124 1.62 Q155R 400 1.27 T325V 635 1.24 D030T 0.57 Q155S 0.77 T325W 0.62 D030V 0.46 Q155T 0.76 I326K 0.95 D030W 0.62 Q155V 0.73 I326L 636 1.50 E031A 125 2.05 Q155W 0.91 I326V 637 6.29 E031C 126 2.95 E156A 0.79 I326Y 0.77 E031G 127 1.27 E156D 401 1.95 L327M 0.52 E031H 128 2.74 E156G 0.49 N328A 0.67 E031I 129 3.89 E156I 0.51 N328C 638 1.25 E031K 130 3.13 E156L 0.43 N328G 639 0.56 E031L 131 2.62 E156M 0.87 N328H 0.88 E031P 132 1.51 E156Q 0.84 N328I 642 1.85 E031R 133 2.27 E156R 0.43 N328K 640 2.12

E031S 134 1.70 E156S 0.62 N328L 641 2.01 E031T 135 3.96 E156T 0.69 N328Q 1.13 E031V 136 4.57 E156V 0.45 N328R 0.68 E031W 137 1.26 E156W 0.49 N328S 643 2.22 E031Y 1.13 F157W 0.61 N328T 0.59 P032A 0.92 E158A 0.56 N328V 1.16 P032C 138 0.40 E158F 0.51 N328Y 644 1.66 P032F 139 2.71 E158H 0.54 I331V 0.94 I326C 0.39 I326S 0.95 N328W 0.33 I331C 0.27 I331E 0.34 V334T 0.39 P032G 140 1.60 E158L 0.44 V334P 0.46 P032H 141 2.08 E158Q 402 1.25 T335S 645 0.47 P032K 1.04 E158S 403 0.95 A338Q 0.63 P032L 0.82 K159A 0.64 K339M 0.61 P032M 0.67 K159D 0.52 S342A 0.68 P032N 0.70 K159E 0.49 Q343T 0.49 P032Q 1.11 K159H 0.74 Q343V 0.51 P032R 1.17 K159L 0.62 Q347A 646 0.78 P032S 1.01 K159M 0.66 Q347E 0.78 P032T 0.77 K159N 0.73 Q347G 647 2.68 P032V 0.81 K159Q 0.92 Q347M 0.61 P032W 0.54 K159R 0.88 Q347R 0.55 P032Y 1.01 K159S 0.67 Q347S 648 2.38 L033G 143 0.57 K159V 0.41 E348D 0.67 L033M 0.69 A160C 0.61 E348G 0.55 L033P 0.87 A160F 0.79 E348S 0.44 L033Q 0.45 A160G 0.75 Q349A 0.47 L033R 0.61 A160H 0.47 Q349E 0.83 L033S 0.48 A160I 0.43 Q349K 0.93 L033T 0.45 A160K 0.91 Q349M 649 0.70 L033W 142 1.58 A160L 0.67 Q349N 0.44 D034A 0.38 M035Q 0.37 M035V 146 0.37 D034E 0.58 A160M 0.77 Q349R 650 0.73 D034H 0.41 A160N 0.56 Q349T 0.49 D034K 0.54 A160Q 0.65 V351A 1.14 D034Q 0.59 A160R 0.89 V351S 651 0.92 D034R 1.17 A160S 404 1.35 I353T 0.42 D034W 144 0.46 A160V 0.73 I353V 652 1.61 M035F 0.87 A160Y 1.07 N356A 0.41 M035H 0.60 G161A 0.99 N356D 0.79 M035L 0.52 G161C 0.44 N356H 653 0.82 M035T 0.83 G161D 0.86 N356S 654 0.46 M035Y 0.78 G161E 0.49 W357A 0.80 S036A 0.45 G161R 0.48 W357C 0.67 S036D 0.32 S036N 148 0.38 L037W 0.36 S036G 0.64 G161S 0.77 W357S 0.41 S036H 147 0.54 G161V 0.42 W357T 0.62 S036K 0.83 K162A 0.50 N358C 0.66 S036L 0.71 K162D 0.77 N358G 0.41 S036R 1.09 K162E 405 0.51 N358T 0.58 Q347L 0.39 V351C 0.35 V351I 0.36 V351Q 0.34 W357K 0.36 N358L 0.38 S036T 0.51 K162G 0.56 S359D 0.45 L037F 149 3.33 K162H 0.62 S359E 655 1.05 L037I 0.62 K162L 0.54 S359H 656 0.44 L037K 0.43 K162M 1.04 S359K 0.66 L037M 150 1.46

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1.16 E167G 0.60 L374H 678 1.42 N047K 0.67 E167H 0.89 L374I 0.80 N047M 0.77 E167K 0.91 L374M 1.11 N047Q 0.69 E167M 0.87 L374N 0.43 N047R 0.84 E167N 0.83 L374P 679 0.43 N047S 0.85 E167P 0.58 L374R 0.83 N047T 162 1.49 E167R 1.02 L374S 0.58 N047W 163 0.63 E167S 1.17 L374T 0.47 N047Y 0.45 E167T 0.59 L374V 0.56 A048F 164 2.51 E167Y 0.55 L374Y 0.66 A048G 0.83 T168H 0.46 E375A 680 0.42 A048H 165 1.99 I169L 430 2.08 E375G 681 0.90 A048I 0.64 I169R 0.54 E375K 682 1.49 A048K 166 1.28 I169V 0.74 E375L 0.46 A048M 0.76 K170N 0.72 E375M 0.54 A048N 167 4.25 K170R 431 2.58 E375N 0.81 A048Q 1.05 K170V 0.58 E375R 683 0.43 A048R 0.66 L171I 0.73 E375S 0.77 A048S 1.06 L171V 0.64 E375T 1.17 A048V 0.60 G172A 432 1.20 K376A 0.95 A048Y 0.81 G172C 1.03 K376D 684 0.78 T049I 0.42 K173N 0.44 K376E 685 0.88 T049K 0.85 K173R 433 0.82 K376M 0.46 T049R 168 1.41 L174A 1.20 K376Q 686 0.69 T049S 0.92 L174G 434 0.40 K376R 687 0.67 T049V 0.45 L174K 435 2.39 K376S 0.80 G050A 0.93 L174M 0.79 K376T 688 0.53 G050C 0.41 L174N 436 1.36 K376V 689 0.58 G050D 169 1.37 L174Q 0.99 K376Y 690 0.42 G050E 0.78 L174R 437 1.50 G377D 691 1.35 G050H 0.74 L174S 0.85 G377E 692 0.59 G050L 0.43 L174T 438 1.12 G377H 693 1.49 G050M 171 0.47 L174V 0.62 G377K 694 1.50 G050Q 0.86 L174W 0.78 G377P 695 2.30 G050R 0.86 L174Y 1.06 G377R 696 1.28 G050S 170 1.24 L175E 0.43 G377S 697 1.80 G050V 0.3 Q051A 0.34 Q051R 0.36 G050Y 0.58 L175H 0.57 G377T 698 3.83 Q051N 0.60 L175T 439 1.43 G378K 1.22 Q051S 0.46 L175V 0.94 G378N 0.64 G052N 172 0.89 L175Y 0.66 G378R 1.03 G052P 0.43 R176K 0.67 K379G 0.52 G052Q 173 3.71 N178G 0.85 K379H 0.57 G052R 174 0.53 N178K 440 0.85 K379R 0.74 G052S 175 1.32 N178M 0.88 K379S 0.46 E375I 0.36 K376L 0.37 K379T 0.4 F380V 0.39 F380T 0.39 M035Q 145 0.37 G052T 176 0.49 N178R 441 1.10 F380I 0.56 T054A 0.43 H179A 1.06 F380L 0.67 T054F 0.56 H179C 0.94 F380P 0.47 T054N 0.48 H179E 0.62 F380W 699 2.15 T054Q 0.91 H179G 0.86 F380Y 700 1.50 T054S 0.70 H179I 0.90 T381H 0.48 T054V 0.66 H179K 442 1.39 T381K 1.06 V058C 177 0.55 H179L 0.73 T381N 0.51 V058G 0.54 H179M 0.63 T381Q 0.84 V058H 183 1.09 H179N 0.96 T381R 0.87 V058I 0.57 H179P 0.44 T381S 701 0.87 V058K 178 4.08 H179R 0.96 T381V 0.89 V058L 179 1.54 H179S 0.51 R383A 0.51 V058N 184 0.49 H179T 0.43 R383E 0.51 V058P 180 0.90 H179V 0.42 R383H 0.71 V058Q 181 4.54 L180F 0.59 R383I 702 0.71 V058R 182 1.92 L180G 0.62 R383K 703 1.30 V058S 0.83 L180K 0.44 R383L 704 1.31 V058W 0.65 L180M 0.64 R383M 0.61 V058Y 185 1.07 W181M 0.88 R383N 0.77 D059Q 0.40 L061F 0.3 T381E 0.35 D059N 186 1.27 W181Q 0.88 R383S 705 0.87 R060K 0.69 G182L 0.90 R383T 0.98 L061I 0.42 Y183L 0.70 R383V 1.05 L061M 0.73 F186Y 0.59 K385A 706 1.12 L061V 0.59 H192S 0.49 K385G 0.62 Y063A 0.63 H192T 0.50 K385H 0.50 Y063H 1.07 H193G 0.68 K385N 0.41 Y063I 1.03 H193Q 443 0.82 K385Q 707 0.73 Y063K 187 1.36 H193S 0.42 K385R 0.94 Y063L 188 1.33 H193Y 0.58 K385S 1.05 Y063M 189 1.32 K195A 0.51 K385T 0.46 Y063N 0.96 K195G 0.45 K385V 708 0.43 Y063R 190 1.40 K195H 0.45 T387S 0.93 Y063S 1.00 K195I 0.50 L388F 0.92 Y063T 1.07 K195L 0.45 L388H 0.47 Y063V 0.43 K195N 445 0.74 L388I 0.98 Y063W 191 1.53 K195Q 0.71 L388M 0.79 P065R 0.57 K195R 0.85 L388R 0.60 Y066H 0.47 K195S 0.42 L388T 0.51 Y066R 0.51 K195T 444 0.58 L388V 0.78 I067F 1.00 K195W 0.49 L388W 0.77 I067L 0.45 K196E 446 0.43 L388Y 1.18 I067R 0.24 D068G 0.37 E392W 0.31 I067V 192 1.80 K196G 0.41 E389A 709 1.14 I067Y 0.55 K196L 0.65 E389G 710 0.91 D068E 0.72 K196R 447 0.58 E389H 1.17 D068H 193 2.06 K196S 0.68 E389K 712 1.91 D068K 1.08 K196T 1.18 E389L 711 0.65 D068L 0.43 K196W 0.55 E389M 0.60 D068P 194 0.50 P197A 0.81 E389P 0.75 D068Q 195 1.67 P197D 0.58 E389Q 713 0.69 D068R 0.70 P197E 0.52 E389R 0.94 D068S 0.81 P197F 0.48 E389S 714 1.08 D068T 0.75 P197G 0.75 E389T 0.70 S069A 196 22.06 P197H 0.62 E389Y 0.77 S069C 197 1.97 P197K 0.99 L391C 0.90 S069E 198

1.48 P197L 0.56 E392A 715 0.58 S069F 199 8.75 P197M 1.03 E392F 716 0.54 S069G 200 6.06 P197Q 0.69 E392G 1.00 S069I 201 3.12 P197R 0.58 E392K 0.66 S069L 202 3.44 P197S 0.70 E392L 0.80 S069M 203 2.67 P197T 0.41 E392M 717 1.54 S069P 204 8.14 G198A 0.80 E392Q 718 1.01 S069R 205 14.06 G198D 448 1.99 E392R 719 0.66 S069T 206 0.58 G198E 0.49 E392S 0.52 S069W 207 2.18 G198H 0.84 E392T 0.72 S069Y 208 2.71 G198L 0.48 E392V 720 1.27 I070A 209 27.00 G198N 0.80 E392Y 0.92 I070C 210 2.57 G198Q 0.55 Q393A 1.26 I070F 211 5.69 G198R 0.58 Q393D 0.45 I070G 212 6.22 G198S 0.76 Q393F 721 1.23 I070H 213 9.09 G198T 0.41 Q393H 1.05 I070K 214 14.64 G198Y 0.81 Q393K 0.80 I070L 215 3.05 N200D 0.46 Q393L 0.91 I070N 216 6.19 S202M 0.40 Q393M 722 0.80 I070P 217 3.03 F204P 449 0.63 Q393N 0.72 I070R 218 13.95 N205A 450 1.30 Q393R 0.74 I070S 219 3.63 N205D 0.85 Q393S 1.15 I070T 220 5.43 N205E 451 1.94 Q393T 0.41 I070V 221 6.34 N205F 0.52 F394L 0.56 I070Y 222 1.26 N205G 0.79 F394W 0.41 T071A 0.86 N205K 0.76 S395A 723 1.10 T071D 0.50 N205M 0.58 S395G 0.77 T071G 223 1.41 N205P 0.75 S395H 724 0.56 T071H 0.93 N205R 0.54 S395K 0.96 T071L 1.09 N205S 0.80 S395R 725 1.98 T071M 0.89 N205T 453 0.85 E396A 726 0.52 T071N 224 1.21 N205V 0.49 E396D 0.64 T071Q 0.68 N205W 0.41 E396H 727 0.47 T071R 225 2.17 V206H 0.50 E396Q 728 0.73 T071S 226 1.54 V2061 454 0.94 E396R 0.61 G072A 0.45 V206K 455 1.75 E396S 729 0.61 G072D 0.60 V206L 456 1.57 E396T 0.89 S395W 0.4 S395T 0.39 E396L 0.39 G072E 0.69 V206M 0.43 Y399A 1.01 G072H 0.46 V206R 457 1.30 Y399C 0.46 G072K 227 1.39 V206S 0.72 Y399E 1.49 G072L 0.43 G072Y 0.35 S407L 0.4 G072M 228 3.11 V206T 0.59 Y399K 730 1.94 G072Q 229 2.33 I208A 0.62 Y399M 731 2.70 G072R 0.65 I208C 0.48 Y399N 0.52 G072S 0.51 I208K 0.91 Y399Q 1.18 V073A 230 1.38 I208L 0.84 Y399R 1.20 V073C 0.84 I208M 0.88 Y399S 1.01 V073D 0.94 I208Q 0.77 Y399T 732 2.40 V073G 1.17 I208R 1.14 Y399V 733 1.44 V073H 231 1.54 I208S 0.62 Y399W 734 1.92 V073K 232 1.42 I208T 1.01 S401A 735 0.82 V073L 233 1.59 I208V 1.07 S401E 736 0.46 V073M 0.68 K209A 0.53 S401N 0.42 V073Q 234 0.96 K209E 0.46 Y403F 0.62 V073R 235 0.72 K209G 0.44 S404A 737 0.63 V073S 0.86 K209N 0.50 S404P 0.64 K297R 0.34 F398L 0.35 S401G 0.38 S401Q 0.39 S404T 0.37 T405F 0.36 V073T 236 1.34 K209R 458 0.68 T405A 0.56 V073W 237 1.91 K209S 0.50 T405G 738 2.32 T074A 238 2.28 K209T 0.50 T405K 0.74 T074C 239 2.18 D212N 459 1.52 T405M 0.48 T074E 240 1.38 D212S 460 0.93 T405P 0.64 T074F 241 1.43 D212T 0.76 T405Q 0.75 T074G 242 2.75 D213A 461 0.85 T405R 0.60 T074H 243 1.40 D213E 0.79 T405S 0.94 T074K 244 1.29 D213G 0.81 T405W 0.73 T074L 245 1.43 D213H 0.75 T405Y 0.44 T074M 246 0.52 D213K 0.82 L406A 0.70 T074N 247 2.12 D213L 0.56 L406C 0.98 T074P 248 2.45 D213M 462 1.56 L406E 0.73 T074R 249 2.22 D213N 463 1.53 L406F 739 1.42 T074S 250 1.80 D213Q 1.04 L406G 1.00 T074V 251 2.27 D213R 0.92 L406I 0.61 T074W 252 2.13 D213V 0.47 L406N 740 0.76 V075A 0.71 D213W 0.49 L406Q 0.93 V075C 0.46 D213Y 0.49 L406S 0.47 V075F 253 2.00 L214Q 0.57 L406T 0.83 V075H 0.62 S215A 0.74 L406V 0.87 V075L 254 5.22 S215D 0.62 L406Y 0.74 V075M 255 1.16 S215E 0.74 S407A 741 1.16 V075N 0.81 S215G 0.88 S407D 742 1.52 V075Q 1.51 S215H 464 0.91 S407E 743 1.38 V075R 256 3.02 S215K 0.99 S407F 744 1.42 V075S 0.76 S215L 0.60 S407G 0.75 V075T 257 4.34 S215M 465 1.77 S407H 745 1.34 V075Y 0.63 S215Q 0.79 S407M 0.74 G077H 0.32 G077K 0.32 K411H 0.33 I079L 258 1.44 S215R 0.71 S407N 0.72 I079T 0.79 S215T 0.80 S407P 747 0.94 I079V 1.01 S215V 0.69 S407Q 746 1.71 Q081P 0.60 S215W 0.52 S407R 1.04 K082A 0.94 W216Y 0.48 S407V 0.56 K082E 0.50 L217M 0.51 S407W 0.41 K082G 0.64 W218F 0.57 K409A 748 2.18 K082H 0.44 N219A 466 1.29 K409D 0.65 K082I 1.01 N219C 0.43 K409E 0.62 K082L 259 0.87 N219D 0.75 K409G 0.50 K082M 0.58 N219E 0.95 K409H 0.64 K082N 260 0.96 N219H 0.97 K409I 0.51 K082Q 0.76 N219I 467 0.60 K409P 0.48 K082R

0.85 N219K 468 1.45 K409Q 749 3.33 K082S 0.62 N219L 0.72 K409R 0.84 K082T 0.56 N219M 1.02 K409S 0.72 K082Y 0.32 I083H 0.4 I083K 0.30 K082V 0.57 N219R 1.10 K409T 0.63 I083F 0.57 N219S 469 2.48 K409V 0.48 I083G 264 1.05 N219T 0.82 A412Y 0.66 I083L 0.93 N219W 0.48 E410D 0.47 I083N 0.82 E220A 0.75 E410K 0.70 I083Q 262 1.07 E220H 470 1.40 E410M 0.42 I083R 0.45 E220I 471 1.34 E410N 0.67 I083S 263 0.79 E220L 472 1.45 E410P 0.73 I083T 0.95 E220S 0.62 E410Q 0.85 I083V 261 0.99 E220T 0.91 E410R 0.61 S084D 0.98 E220V 473 1.35 E410S 0.81 S084E 265 0.52 S221A 0.72 E410T 750 1.54 S084F 266 0.72 S221C 0.59 E410V 0.65 S084G 267 8.68 S221M 0.46 E410Y 0.62 S084H 0.96 S221Q 474 1.37 K411A 0.48 S084I 0.90 S221T 0.94 K411N 1.02 S084L 0.92 S221V 1.04 K411P 0.42 S084M 0.77 T222D 0.43 K411R 0.97 S084N 268 0.89 T222F 0.43 K411S 1.21 S084P 0.57 T222G 475 0.49 K411T 0.63 S084Q 0.86 T222K 0.75 K411V 0.99 S084R 269 1.89 T222L 0.64 A412D 0.74 S084T 0.82 T222N 0.80 A412G 0.80 S084W 0.86 T222R 0.75 A412I 0.81 S084Y 0.30 E220D 0.39 E220M 0.36 S221I 0.35 T222I 0.4 P226W 0.51 L085V 0.42 T222S 0.63 A412L 0.65 Q086A 270 2.70 T222V 0.79 A412N 0.86 Q086D 0.88 L224I 0.61 A412P 0.77 Q086E 1.18 L230I 0.87 A412R 752 0.66 Q086F 0.54 N231T 1.10 A412S 0.86 Q086G 1.02 T232F 476 0.73 A412V 753 0.53 Q086H 271 1.70 T232S 0.76 A412W 0.54 Q086I 0.65 Q233A 0.71 D413E 0.52 Q086K 272 0.97 Q233F 0.53 D413K 0.42 Q086L 0.92 Q233G 477 0.46 D413N 0.94 Q086M 1.06 Q233K 478 1.69 D413R 0.50 Q086N 273 1.28 Q233L 0.69 D413T 0.41 Q086P 0.42 Q233R 479 1.50 V414I 1.12 Q086R 0.93 Q233Y 0.50 V414M 0.53 Q086S 274 0.85 Q234M 480 1.65 K415G 0.40 Q086T 275 0.58 S235A 481 0.47 K415S 0.42 Q086V 0.97 S235E 1.00 K415W 0.42 Q086W 276 1.21 S235G 0.95 D416F 0.41 D087A 1.00 S235H 0.44 D416G 0.67 D087C 277 1.77 S235K 0.53 D416H 0.57 D087E 0.86 S235T 0.66 D416I 0.63 D087G 278 1.00 P236A 1.07 D416K 0.76 D087H 0.72 P236G 1.09 D416L 754 0.75 D087I 0.53 P236H 0.46 D416N 0.73 D087L 279 0.55 P236K 0.71 D416Q 0.83 D087M 280 0.58 P236R 482 3.09 D416R 0.46 D087P 0.31 Q234L 0.40 V237C 483 0.35 D087Q 1.05 P236S 0.91 D416T 0.85 D087R 281 1.28 V237A 0.90 D416V 0.59 D087S 282 0.99 V237E 484 1.93 D416Y 0.40 D087T 283 1.70 V237F 0.41 T417I 1.22 A412H 0.39 A412Q 751 0.35 D413A 0.38 D413H 0.31 A413Q 0.38 D413S 0.39 V414K 0.3 V414L 0.36 K415Y 0.39 K415V 0.39 D418G 0.45 D087V 284 0.66 V237H 485 0.75 D418A 0.92 D087Y 285 2.72 V237L 1.12 D418E 755 1.31 L089C 286 1.46 V237N 0.67 D418F 0.81 L089R 0.34 L089W 0.26 L089P 0.38 L089K 0.45 V237Q 486 1.46 D418G 0.45 L089M 0.63 V237R 0.71 D418I 0.99 D090A 287 1.48 V237S 1.03 D418L 756 1.28 D090E 288 1.15 V237T 487 1.01 D418M 1.09 D090G 0.41 V237W 0.52 D418N 0.91 D090H 289 1.24 A238D 0.75 D418P 757 2.11 D090I 1.10 A238E 488 0.59 D418Q 1.05 D090K 290 1.36 A238H 489 0.60 D418R 758 1.18 D090L 1.15 A238K 0.60 D418S 0.78 D090N 291 1.18 A238Q 1.02 D418V 759 1.43 D090Q 1.11 A238R 0.49 D418Y 0.97 D090R 292 1.49 A238S 490 2.62 A419E 0.45 D090S 1.15 A238T 0.44 A419F 760 2.17 D090T 1.02 T240K 1.13 A419G 0.42 D090W 0.81 T240A 491 0.48 A419H 761 1.21 K091A 0.89 T240M 0.48 A419I 762 1.64 K091Q 0.43 T240P 0.56 A419K 763 1.88 K091R 0.67 T240Q 492 0.75 A419L 0.56 A092C 293 1.97 T240R 0.91 A419N 0.53 A092H 0.22 A239N 0.32 V421I 0.39 A092L 294 1.29 T240S 0.74 A419R 764 1.81 A092M 0.86 T240V 0.77 A419S 765 2.65 A092T 0.70 Y242F 1.08 A419W 0.69 A092V 1.09 N245H 0.50 A419Y 766 1.44 K093D 0.71 V247I 493 2.01 V420I 1.04 K093E 0.83 V247L 0.83 V420P 0.48 K093F 0.50 V247M 0.52 D421A 767 1.28 K093G 0.97 R248A 494 0.43 D421E 0.81 K093H 0.61 R248W 0.52 D421G 0.62 K093I 295 3.25 R248Y 0.67 D421H 768 1.98 R248H 0.4 I251Y 0.37 K255G 0.39 K093L 296 1.53 I251L 0.58 D421K 769 2.42 K093M 0.70 I251M 0.43 D421L 0.73 K093N 0.71 V253I 0.76 D421M 0.94 K093Q 297 0.84 K255A 0.40 D421N 770 1.89 K093R 298 1.52 K255N 0.52 D421Q 771 1.54 K093S 299

1.25 K255Q 0.91 D421R 772 2.21 K093T 300 3.93 K255R 0.71 D421S 773 2.12 K093V 0.24 K093P 0.38 K094C 0.33 K094A 0.64 K255S 0.43 D421T 0.80 K094D 301 0.93 I256A 0.42 D421Y 0.66 K094E 0.79 I256H 0.51 V422I 0.42 K094F 0.59 I256L 0.64 V422T 0.49 K094H 0.72 I256V 0.51 A425G 774 1.20 K094L 0.52 P257A 0.82 A425I 0.44 K094M 0.66 P257G 496 0.51 A425K 775 1.75 K094N 0.99 P257I 1.07 A425M 0.70 K094Q 302 1.22 P257K 0.92 A425N 0.46 K094R 303 3.94 P257L 0.69 A425R 0.49 K094S 0.94 P257M 0.90 A425S 0.47 K094T 1.14 P257N 0.69 D426E 0.62 I096D 0.69 P257Q 0.61 D426G 0.85 I096L 0.46 P257R 498 1.38 D426N 0.61 I096V 0.68 P257T 497 2.04 D426P 1.03 T097A 304 1.25 P257V 0.88 D426Q 0.42 T097C 305 0.53 D258H 0.84 D426Y 0.43 T097D 306 1.31 D258N 499 1.44 G427K 0.52 T097E 307 1.19 D258R 0.45 G427S 0.42 T097F 0.75 D258S 500 1.44 V428L 778 1.25 P257C 0.36 D258G 0.39 A425Y 0.39 D426K 0.26 D426S 0.36 G427T 777 0.35 G427H 0.35 G427I 0.54 G427Q 776 0.39 T097G 308 4.84 A259E 0.85 V428M 0.42 T097I 0.85 A259G 0.68 V428P 0.82 T097L 309 1.22 A259I 0.46 V428T 0.62 T097N 1.10 A259K 0.76 D431A 779 2.42 T097P 0.62 A259L 0.53 D431E 781 1.27 T097Q 1.17 A259N 0.49 D431G 780 0.55 T097R 0.95 A259P 501 1.54 D431H 782 3.13 T097S 310 1.21 ~~T097W 0.53 T097Y 0.74 F098A 0.60 F098C 0.58 F098D 0.47 F098E 0.44 F098H 1.06 F098I 0.52 F098L 0.58 F098M 0.87 F098Q 0.65 P436C 0.39 F098R 0.72 F098S 0.56 F098V 0.46 F098W 0.81 Y099A 0.33 Y099R 0.53 Y099S 0.43 V102A 0.83 V102C 0.69 V102E 0.90 V102G 0.67 V102H 0.88 V102K 1.03 V102L 0.71 V102M 0.77 V102N 1.02 V102Q 1.03 V102R 0.94 V102S 311 1.41 V102T 312 1.26 V102W 0.76 D103N 0.39 N104A 0.69 N104C 0.41 N104G 0.48 N104K 0.88 N104M 0.61 N104R 313 1.25 N104S 1.03 N104T 0.71 L105A 0.54 L105G 0.51 L105I 0.94 L105P 0.84 L105Q 0.90 L105R 0.65 L105S 0.61 L105T 0.51 L105W 0.34 L105V 0.99 G106V 0.43 M107F 0.91 M107I 0.67 M107L 314 1.32 A108G 0.47 I110V 0.51 E114A 315 1.44 E114G 0.73 E114H 0.75 E114M 0.44 E114S 0.69 P117D 0.56 T118H 0.47 T118K 0.53 T118L 1.09 T118M 0.53 T118N 0.67 T118Q 316 3.37 T118V 0.79 W119F 0.53 W119P 0.36 W119Y 1.08 A120D 0.76 A120F 318 2.62 A120G 1.03 A120H 317 1.11 A120I 319 1.33 A120L 1.25 A120N 0.81 A120P 0.42 A120R 0.82 A120S 320 1.21 A120T 0.62 A120V 321 1.53 A120W 0.59 A120Y 322 1.95 N122M 0.56 K124L 0.34 K124R 0.62 P125H 0.43 P125R 0.63 P125S 0.54 D127A 0.89 D127E 323 1.31 D127G 0.97 D127H 324 2.33 D127L 0.84 D127M 0.4 D127N 325 1.69 D127Q 326 1.21 D127R 327 0.51 D127S 0.77 D127T 1.11 D127V 0.56 D127W 0.44 V128A 0.53 V128C 0.68 V128G 0.49 V128I 328 1.25 V128K 1.16~~

A259Q 0.70 D431I 1.05 T097W 0.53 A259R 0.72 D431K 783 1.83 T097Y 0.74 A259S 0.63 D431L 784 0.62 F098A 0.60 A259T 0.51 D431N 785 1.30 F098C 0.58 A259V 0.41 D431Q 786 2.16 F098D 0.47 A259W 0.55 D431R 787 2.20 F098E 0.44 A259Y 0.51 D431S 788 1.91 F098H 1.06 K260A 0.66 D431V 789 1.52 F098I 0.52 K260D 0.41 D431W 0.56 F098L 0.58 K260E 0.58 D431Y 0.85 F098M 0.87 K260H 0.87 A432E 0.60 F098Q 0.65 K260L 0.60 A432G 0.52 P436C 0.39 E249V 495 A432H 0.34 F098R 0.72 K260M 502 0.85 A432N 0.51 F098S 0.56 K260Q 0.58 A432S 0.61 F098V 0.46 K260R 0.83 A432V 0.56 F098W 0.81 K260S 0.66 F433A 790 0.97 Y099A 0.33 K260G 0.37 R270T 0.40 Y099R 0.53 K260Y 503 1.73 F433C 0.69 Y099S 0.43 S261A 504 0.74 F433D 0.95 V102A 0.83 ~~V128L 0.95 V128Q 0.55 V128R 0.74 V128S 0.53 V128W 0.50 K130I 0.50 K130R 329 1.42 N131C 0.60 N131E 0.44 N131F 0.63 N131G 330 2.47 N131H 0.80 N131I 331 1.40 N131L 0.82 N131M 332 0.99 N131Q 333 1.24 N131R 334 2.81 N131S 0.76 N131T 1.02 N131V 335 2.08 N131Y 0.85 R132A 0.68 R132C 0.58 R132E 0.70 R132F 0.60 R132H 0.66 K279A 0.27 E285A 0.34 R132I 0.56 R132K 1.05 R132L 337 0.76 R132N 336 1.28 R132Q 0.69 R132S 0.79 R132T 0.61 R132V 0.73 R132Y 0.78 S133I 0.54 I134L 1.04 I134T 0.60 I134V 1.08 E135A 0.99 E135C 0.77 E135D 338 2.68 E135F~~

0.73 E442L 0.4 E135G 339 2.79 E135H 0.79 E135K 1.15 E135L 0.82 E135N 0.56 E135Q 1.59 E135R 340 2.08 E135S 1.13 E135W 0.63 E135Y 0.50 L136A 0.73 L136C 0.56 L136D 0.47 L136F 0.96 L136H 1.00 L136I 0.65 L136M 1.05 L136N 0.48 L136Q 0.61 L136R 0.74 L136S 0.80 L136T 0.72 L136W 1.11 V137A 0.48 V137I 1.01 V137T 0.51 Q138A 0.69 Q138C 0.65 Q138H 0.71 Q138I 0.54 Q138L 341 0.59 Q138M 0.68 Q138N 0.61 Q138R 0.53 Q138S 0.48 Q138W 0.41 Q138Y 0.60 Q139A 0.92 Q139C 0.44 Q139D 0.48 Q139E 0.94 Q139F 0.53 Q139G 0.65 Q139H 0.56 Q139K 0.73 Q139L 0.70 Q139M 0.95 Q139R 0.79 Q139S 0.81 Q139T 342 1.31 Q139V 0.77 Q140A 0.96 Q140C 0.50 Q140D 0.59 Q140F 0.66 Q140G 0.73 Q140H 0.84 Q140I 0.75 Q140K 343 0.93 Q140L 0.51 Q140M 0.80 Q140R 0.85 Q140V 0.61 Q140W 0.59 Q140Y 0.41 N141A 1.12 N141D 1.09 N141E 0.67 N141F 0.81 N141G 1.15 N141H 344 2.03 N002I 0.37 G297A 0.57 N141L 0.61 N141M 0.48 N141Q 1.16 N141R 345 1.40 N141S 346 0.72 N141T 0.45 N141V 0.50 N141W 347 0.83 N141Y 348 1.55 V142C 0.61 V142D 349 0.71 V142E 0.87 V142G 350 0.98 V142H 1.11 V142I 0.81 V142K 351 1.40 V142L 0.75 V142M 0.76 V142N 352 0.98 V142P 353 0.88 V142Q 354 1.04 V142R 355 1.53 V142S 356 0.93 V142T 357 1.19 Q143E 0.77 Q143G 358 0.62 Q143I 0.44 Q143K 359 1.30 I009Q 0.4 Q143L 0.56 Q143N 0.73 Q143V 0.57 L144T 361 1.02 L144W 0.79 S145A 0.58 S145C 0.44 S145D 0.48 S145E 0.56 S145G 0.94 S145H 0.56 S145L 0.44 S145M 0.56 S145N 0.58 S145P 1.04 S145R 0.97 L146A 0.52 L146C 0.42 G305N 0.36 M310Y 0.38 L146E 0.50 L146G 0.62 L146H 0.78 L146I 0.82 L146K 0.84 L146N 0.57 L146P 362 0.93 L146Q 0.84 L146R 363 1.47 L146S 0.71 L146T 0.74 L146V 0.84 L146Y 0.80 S312K 0.38 T147A 364 1.20 T147C 0.47 T147D 0.71 T147F 365 1.24 T147G 1.05 T147I 0.85 T147L 366 1.30 T147M 0.79 T147P 1.09 T147Q 1.29 T147R 367 2.11 T147S 368 1.27 T147V 369 2.04 T147W 0.97 T147Y 1.04 E148C 0.66 E148F 0.42 E148G 1.05 E148H 370 1.24 E148I 0.73 E148K 371 1.63 E148L 0.85 E148Q 372 1.44 E148R 0.97 E148S 1.15 E148T 0.82 E148V 0.99 E148W 0.43 E148Y 0.95 A149C 1.15 A149G 0.52 A149K 0.51 A149L 0.88 A149M 0.88 A149Q 1.15 A149R 1.02 A149S 1.08 A149T 373 1.24 A149V 374 1.34 T150A 375 1.21 T150C 0.70 T150D 376 1.24 T150E 1.05 T150F 0.71 T150G 377 2.19 T150I 0.52 T150L 0.70 T150N 378 0.91 T150P 0.88 T150R 0.90 T150S 379 0.92 T150W 380 1.25 T150Y 381 1.36 E151A 382 1.27 E151C 1.00 E151G 1.06 E151H 383 1.34 E151K 384 2.05 E151L 385 1.03 E151M 386 1.26 E151N 0.95 E151Q 387 2.01 D320L 0.37 E151R 388 1.61 E151S 389 1.28 E151T 390 1.21 E151V 391 1.38 E151W 392 1.31 E151Y 393 1.31

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D163R 410 1.80 D163S 411 1.34 D163T 1.13 D163V 0.76 F164L 1.13 F164M 412 1.66 F164V 413 1.23 S043N 0.34 F164W 0.72 L165A 0.48 L165D 414 5.79 L165F 415 1.23 L165N 416 2.19 L165R 0.59 L165S 417 1.31 L165V 418 1.22 L165W 1.14 A371G 0.38 L165Y 0.66 V166A 419 2.85 V166C 1.16 V166E 420 1.28 V166F 421 1.67 V166G 1.11 V166H 422 1.74 V166L 423 4.38 V166Q 424 3.61 V166R 425 5.56 V166T 426 4.26 V166W 427 1.26 V166Y 428 2.08 E167A 0.84 E167D 429 0.69 E167G 0.60 E167H 0.89 E167K 0.91 E167M 0.87 E167N 0.83 E167P 0.58 E167R 1.02 E167S 1.17 E167T 0.59 E167Y 0.55 T168H 0.46 I169L 430 2.08 I169R 0.54 I169V 0.74 K170N 0.72 K170R 431 2.58 K170V 0.58 L171I 0.73 L171V 0.64 G172A 432 1.20 G172C 1.03 K173N 0.44 K173R 433 0.82 L174A 1.20 L174G 434 0.40 L174K 435 2.39 L174M 0.79 L174N 436 1.36 L174Q 0.99 L174R 437 1.50 L174S 0.85 L174T 438 1.12 L174V 0.62 L174W 0.78 L174Y 1.06 L175E 0.43 Q051A 0.34 L175H 0.57 L175T 439 1.43 L175V 0.94 L175Y 0.66 R176K 0.67 N178G 0.85 N178K 440 0.85 N178M 0.88 K376L 0.37 F380T 0.39 N178R 441 1.10 H179A 1.06 H179C 0.94 H179E 0.62 H179G 0.86 H179I 0.90 H179K 442 1.39 H179L 0.73 H179M 0.63 H179N 0.96 H179P 0.44 H179R 0.96 H179S 0.51 H179T 0.43 H179V 0.42 L180F 0.59 L180G 0.62 L180K 0.44 L180M 0.64 W181M 0.88 L061F 0.3 W181Q 0.88 G182L 0.90 Y183L 0.70 F186Y 0.59 H192S 0.49 H192T 0.50 H193G 0.68 H193Q 443 0.82 H193S 0.42 H193Y 0.58 K195A 0.51 K195G 0.45 K195H 0.45 K195I 0.50 K195L 0.45 K195N 445 0.74 K195Q 0.71 K195R 0.85 K195S 0.42 K195T 444 0.58 K195W 0.49 K196E 446 0.43 D068G 0.37 K196G 0.41 K196L 0.65 K196R 447 0.58 K196S 0.68 K196T 1.18 K196W 0.55 P197A 0.81 P197D 0.58 P197E 0.52 P197F 0.48 P197G 0.75 P197H 0.62 P197K 0.99 P197L 0.56 P197M 1.03 P197Q 0.69 P197R 0.58 P197S 0.70 P197T 0.41 G198A 0.80 G198D 448 1.99 G198E 0.49

G198H 0.84 G198L 0.48 G198N 0.80 G198Q 0.55 G198R 0.58 G198S 0.76 G198T 0.41 G198Y 0.81 N200D 0.46 S202M 0.40 F204P 449 0.63 N205A 450 1.30 N205D 0.85 N205E 451 1.94 N205F 0.52 N205G 0.79 N205K 0.76 N205M 0.58 N205P 0.75 N205R 0.54 N205S 0.80 N205T 453 0.85 N205V 0.49 N205W 0.41 V206H 0.50 V206I 454 0.94 V206K 455 1.75 V206L 456 1.57 S395T 0.39 V206M 0.43 V206R 457 1.30 V206S 0.72 G072Y 0.35 V206T 0.59 I208A 0.62 I208C 0.48 I208K 0.91 I208L 0.84 I208M 0.88 I208Q 0.77 I208R 1.14 I208S 0.62 I208T 1.01 I208V 1.07 K209A 0.53 K209E 0.46 K209G 0.44 K209N 0.50 F398L 0.35 S404T 0.37 K209R 458 0.68 K209S 0.50 K209T 0.50 D212N 459 1.52 D212S 460 0.93 D212T 0.76 D213A 461 0.85 D213E 0.79 D213G 0.81 D213H 0.75 D213K 0.82 D213L 0.56 D213M 462 1.56 D213N 463 1.53 D213Q 1.04 D213R 0.92 D213V 0.47 D213W 0.49 D213Y 0.49 L214Q 0.57 S215A 0.74 S215D 0.62 S215E 0.74 S215G 0.88 S215H 464 0.91 S215K 0.99 S215L 0.60 S215M 465 1.77 S215Q 0.79 G077K 0.32 S215R 0.71 S215T 0.80 S215V 0.69 S215W 0.52 W216Y 0.48 L217M 0.51 W218F 0.57 N219A 466 1.29 N219C 0.43 N219D 0.75 N219E 0.95 N219H 0.97 N219I 467 0.60 N219K 468 1.45 N219L 0.72 N219M 1.02 I083H 0.4 N219R 1.10 N219S 469 2.48 N219T 0.82 N219W 0.48 E220A 0.75 E220H 470 1.40 E220I 471 1.34 E220L 472 1.45 E220S 0.62 E220T 0.91 E220V 473 1.35 S221A 0.72 S221C 0.59 S221M 0.46 S221Q 474 1.37 S221T 0.94 S221V 1.04 T222D 0.43 T222F 0.43 T222G 475 0.49 T222K 0.75 T222L 0.64 T222N 0.80 T222R 0.75 E220D 0.39 T222I 0.4 T222S 0.63 T222V 0.79 L224I 0.61 L230I 0.87 N231T 1.10 T232F 476 0.73 T232S 0.76 Q233A 0.71 Q233F 0.53 Q233G 477 0.46 Q233K 478 1.69 Q233L 0.69 Q233R 479 1.50 Q233Y 0.50 Q234M 480 1.65 S235A 481 0.47 S235E 1.00 S235G 0.95 S235H 0.44 S235K 0.53 S235T 0.66 P236A 1.07 P236G 1.09 P236H 0.46 P236K 0.71 P236R 482 3.09 Q234L 0.40 P236S 0.91 V237A 0.90 V237E 484 1.93 V237F 0.41 A412Q 751 0.35 A413Q 0.38 V414L 0.36 D418G 0.45 V237H 485 0.75 V237L 1.12 V237N 0.67 L089W 0.26 V237Q 486 1.46 V237R 0.71 V237S 1.03 V237T 487 1.01 V237W 0.52

~~A238D 0.75 A238E 488 0.59 A238H 489 0.60 A238K 0.60 A238Q 1.02 A238R 0.49 A238S 490 2.62 A238T 0.44 T240K 1.13 T240A 491 0.48 T240M 0.48 T240P 0.56 T240Q 492 0.75 T240R 0.91 A239N 0.32 T240S 0.74 T240V 0.77 Y242F 1.08 N245H 0.50 V247I 493 2.01 V247L 0.83 V247M 0.52 R248A 494 0.43 R248W 0.52 R248Y 0.67 I251Y 0.37 I251L 0.58 I251M 0.43 V253I 0.76 K255A 0.40 K255N 0.52 K255Q 0.91 K255R 0.71 K093P 0.38 K255S 0.43 I256A 0.42 I256H 0.51 I256L 0.64 I256V 0.51 P257A 0.82 P257G 496 0.51 P257I 1.07 P257K 0.92 P257L 0.69 P257M 0.90 P257N 0.69 P257Q 0.61 P257R 498 1.38 P257T 497 2.04 P257V 0.88 D258H 0.84 D258N 499 1.44 D258R 0.45 D258S 500 1.44 D258G 0.39 D426S 0.36 G427I 0.54 A259E 0.85 A259G 0.68 A259I 0.46 A259K 0.76 A259L 0.53 A259N 0.49 A259P 501 1.54 A259Q 0.70 A259R 0.72 A259S 0.63 A259T 0.51 A259V 0.41 A259W 0.55 A259Y 0.51 K260A 0.66 K260D 0.41 K260E 0.58 K260H 0.87 K260L 0.60 E249V K260M 502 0.85 K260Q 0.58 K260R 0.83 K260S 0.66 K260G 0.37 K260Y 503 1.73 S261A 504 0.74~~

S261F 0.73 F433E 0.82 V102C 0.69 S261K 505 2.54 F433G 0.54 V102E 0.90 S261M 0.56 F433H 791 0.83 V102G 0.67 S261N 506 1.98 F433I 792 1.06 V102H 0.88 S261Q 0.76 F433K 793 1.36 V102K 1.03 S261R 1.19 F433L 794 1.87 V102L 0.71 S261T 0.66 F433P 0.95 V102M 0.77 S261V 0.48 F433R 795 1.63 V102N 1.02 S261W 0.44 F433S 0.86 V102Q 1.03 L263A 0.76 F433T 796 1.86 V102R 0.94 L263K 507 2.73 F433V 797 1.63 V102S 311 1.41 L263M 0.89 F433W 798 1.28 V102T 312 1.26 L263R 508 1.63 L434F 0.41 V102W 0.76 L263T 0.49 L434G 0.47 D103N 0.39 N104I 0.35 L263H 0.36 N104A 0.69 L263V 0.75 L434I 0.89 N104C 0.41 P264A 0.43 L434M 0.60 N104G 0.48 P264H 0.60 L434V 0.46 N104K 0.88 V265I 0.58 K435A 1.08 N104M 0.61 F266Y 0.58 K435C 0.53 N104R 313 1.25 A267M 0.45 K435E 0.78 N104S 1.03 A267T 509 1.34 K435G 0.64 N104T 0.71 T269A 510 1.63 K435H 1.05 L105A 0.54 T269C 0.75 K435R 1.01 L105G 0.51 T269D 0.76 K435S 1.03 L105I 0.94 T269S 1.01 K435T 0.73 L105P 0.84 R270M 0.46 K435V 0.44 L105Q 0.90 R270N 0.52 K435Y 0.50 L105R 0.65 R270S 0.69 P436D 1.19 L105S 0.61 I271F 0.72 P436E 0.74 L105T 0.51 I271G 1.29 P436G 1.19 L105W 0.34 L105C 0.33 L105H 0.36 L105V 0.99 I271L 511 10.62 P436H 0.72 G106V 0.43 V272E 0.39 V272M 0.31 M107F 0.91 I271M 512 3.24 P436I 0.84 M107I 0.67 I271S 0.42 P436K 799 2.05 M107L 314 1.32 I271V 1.05 P436L 0.63 A108G 0.47 V272D 513 1.36 P436M 0.61 I110V 0.51 V272R 0.74 P436Q 0.86 E114A 315 1.44 V272S 0.96 P436R 1.00 E114G 0.73 V272T 514 1.61 P436S 0.92 E114H 0.75 F273H 515 1.41 P436T 0.59 E114M 0.44 F273T 0.48 P436W 0.43 E114S 0.69 F273Y 516 0.90 P436Y 0.49 P117D 0.56 T274A 0.51 P437A 0.56 T118H 0.47 T274F 517 1.28 P437D 0.62 T118K 0.53 T274S 0.62 P437G 0.50 T118L 1.09 Q276C 0.88 P437H 1.11 T118M 0.53 Q276D 518 1.69 P437I 800 2.46 T118N 0.67 Q276E 1.05 P437K 0.83 T118Q 316 3.37 Q276H 519 1.20 P437L 0.51 T118V 0.79 Q276I 0.51 P437M 801 2.55 W119F 0.53 Q276L 0.48 P437Q 0.96 W119P 0.36 W119Q 0.72 D275L 0.24 W119Y 1.08 Q276M 520 1.14 P437R 0.85 A120D 0.76 Q276R 521 1.30 P437S 0.57 A120F 318 2.62 Q276S 522 1.63 P437Y 0.42 A120G 1.03 Q276Y 523 1.94 M438A 802 0.75 A120H 317 1.11 V277A 524 0.65 M438C 0.63 A120I 319 1.33 V277C 0.41 M438D 803 0.87 A120L 1.25 V277D 0.79 M438E 804 0.72 A120N 0.81 V277E 525 1.02 M438G 0.83 A120P 0.42 V277G 1.18 M438L 805 0.86 A120R 0.82 V277H 526 1.09 M438N 806 1.08 A120S 320 1.21 V277K 527 1.51 M438P 0.81 A120T 0.62 V277M 528 0.94 M438Q 0.85 A120V 321 1.53 V277N 529 1.15 M438R 0.99 A120W 0.59 V277Q 530 0.82 M438S 0.83 A120Y 322 1.95 V277R 531 1.63 M438T 807 3.99 N122M 0.56 V277S 532 0.83 M438V 0.85 K124L 0.34 K124H 0.35 P125A 0.36 K124R 0.62 V277T 533 1.94 M438W 0.57 P125H 0.43 V277Y 0.66 E439A 808 1.20 P125R 0.63 L278A 1.13 E439C 809 0.58 P125S 0.54 L278E 534 1.03 E439F 1.00 D127A 0.89 L278F 535 1.26 E439G 1.22 D127E 323 1.31 L278G 536 1.33 E439H 0.74 D127G 0.97 L278H

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T440D 815 1.03 V128A 0.53 K279Q 0.84 T440E 1.00 V128C 0.68 K279R 1.10 T440F 0.85 V128G I309W 0.88 M310A 587 1.50 M310G 588 2.73 M310Q 589 0.59 M310R 0.50 M310S 590 1.61 M310V 0.70 R311G 0.53 L307G 570 0.32 R311G 0.54 R311H 0.48 R311K 0.72 R311Q 0.43 R311S 0.84 R311T 0.52 S312G 0.49 S312N 1.26 S312T 0.75 M313A 591 1.34 M313E 0.63 M313G 592 0.56 M313H 593 1.23 M313K 594 2.85 S312L 0.38 M313L 1.05 M313P 595 1.11 M313R 596 2.30 M313S 0.88 M313T 597 0.67 M313V 0.99 M313Y 598 1.12 K314A 0.82 K314D 0.53 K314H 1.10 K314I 0.54 K314N 0.57 K314Q 0.62 K314R 0.95 K314S 599 0.61 K314T 0.61 K314Y 600 0.45 S315A 601 0.85 S315E 0.41 S315G 0.72 S315H 602 2.04 S315K 0.62 S315L 0.42 S315M 0.63 S315R 1.04 S315T 0.97 S315Y 603 0.50 C316D 0.41 L317A 604 1.27 L317D 0.61 L317H 1.05 L317I 605 1.76 L317K 606 5.11 L317M 1.20 L317N 607 0.73 L317Q 608 1.67 L317R 609 2.41 L317S 610 1.03 L317T 611 0.93 L317W 612 0.84 L318D 614 0.46 L318F 0.51 L318G 0.49 L318H 615 0.45 L318I 0.70 L318K 616 1.36 L318M 613 1.68 L318N 0.52 L318Q 0.71 L318R 617 1.34 L318S 0.71 L318T 0.63 D320E 0.78 D320G 0.83 D320H 618 1.75 D320I 1.00 D320K 619 6.42 D320M 0.79 D320N 0.52 D320R 620 3.19 D320S 1.19 D320W 0.40 D320V 0.35 D320Y 0.86 N321A 1.01 N321D 1.25 N321H 0.92 N321K 1.29 N321R 621 1.23 N321S 622 1.26 N321T 0.64 N321Y 0.40 M323F 0.64 M323I 0.55 M323L 0.55 E324A 0.59 E324D 1.15 E324H 0.79 E324M 0.50 E324N 623 1.01 E324R

624 2.28 E324S 0.62 T325A 625 1.87 T325D 626 1.78 T325E 627 4.03 T325G 628 4.21 T325H 629 3.45 T325K 630 4.37 T325M 631 2.11 T325N 632 4.64 T325Q 633 5.08 T325S 634 3.19 T325V 635 1.24 T325W 0.62 I326K 0.95 I326L 636 1.50 I326V 637 6.29 I326Y 0.77 L327M 0.52 N328A 0.67 N328C 638 1.25 N328G 639 0.56 N328H 0.88 N328I 1.85 N328K 640 2.12 N328L 641 2.01 N328Q 1.13 N328R 0.68 N328S 643 2.22 N328T 0.59 N328V 1.16 N328Y 644 1.66 I331V 0.94 N328W 0.33 V334T 0.39 V334P 0.46 T335S 645 0.47 A338Q 0.63 K339M 0.61 S342A 0.68 Q343T 0.49 Q343V 0.51 Q347A 646 0.78 Q347E 0.78 Q347G 647 2.68 Q347M 0.61 Q347R 0.55 Q347S 648 2.38 E348D 0.67 E348G 0.55 E348S 0.44 Q349A 0.47 Q349E 0.83 Q349K 0.93 Q349M 649 0.70 Q349N 0.44 M035V 0.37 Q349R 650 0.73 Q349T 0.49 V351A 1.14 V351S 651 0.92 I353T 0.42 I353V 652 1.61 N356A 0.41 N356D 0.79 N356H 653 0.82 N356S 654 0.46 W357A 0.80 W357C 0.67 L037W 0.36 W357S 0.41 W357T 0.62 N358C 0.66 N358G 0.41 N358T 0.58 V351I 0.36 N358L 0.38 S359D 0.45 S359E 655 1.05 S359H 656 0.44 S359K 0.66 S359M 0.63 S359T 657 2.11 S359V 0.65 S360T 0.50 P367A 658 0.55 P367C 0.83 P367G 659 0.47 P367K 660 0.57 P367R 0.46 P367S 661 0.52 D368A 662 1.34 D368E 663 1.28 D368G 0.49 D368H 0.96 D368K 664 1.31 D368L 665 0.64 D368M 666 0.78 D368R 667 1.31 D368S 0.93 D368T 668 0.80 D361H 0.37 D368V 0.41 N369H 669 1.33 N369R 670 0.55 N369S 0.54 A371E 1.05 A371F 671 0.52 A371H 672 1.20 A371I 0.50 A371K 673 1.76 L374W 0.34 A371L 674 0.57 A371M 0.57 A371R 675 1.51 A371S 676 1.45 A371V 0.94 Q373A 0.65 Q373E 0.81 Q373F 0.62 Q373K 0.73 Q373L 0.84 Q373M 677 1.43 Q373R 0.68 Q373S 0.87 Q373V 1.05 L374A 0.60 L374H 678 1.42 L374I 0.80 L374M 1.11 L374N 0.43 L374P 679 0.43 L374R 0.83 L374S 0.58 L374T 0.47 L374V 0.56 L374Y 0.66 E375A 680 0.42 E375G 681 0.90 E375K 682 1.49 E375L 0.46 E375M 0.54 E375N 0.81 E375R 683 0.43 E375S 0.77 E375T 1.17 K376A 0.95

K376D 684 0.78 K376E 685 0.88 K376M 0.46 K376Q 686 0.69 K376R 687 0.67 K376S 0.80 K376T 688 0.53 K376V 689 0.58 K376Y 690 0.42 G377D 691 1.35 G377E 692 0.59 G377H 693 1.49 G377K 694 1.50 G377P 695 2.30 G377R 696 1.28 G377S 697 1.80 Q051R 0.36 G377T 698 3.83 G378K 1.22 G378N 0.64 G378R 1.03 K379G 0.52 K379H 0.57 K379R 0.74 K379S 0.46 K379T 0.4 M035Q 145 0.37 F380I 0.56 F380L 0.67 F380P 0.47 F380W 699 2.15 F380Y 700 1.50 T381H 0.48 T381K 1.06 T381N 0.51 T381Q 0.84 T381R 0.87 T381S 701 0.87 T381V 0.89 R383A 0.51 R383E 0.51 R383H 0.71 R383I 702 0.71 R383K 703 1.30 R383L 704 1.31 R383M 0.61 R383N 0.77 T381E 0.35 R383S 705 0.87 R383T 0.98 R383V 1.05 K385A 706 1.12 K385G 0.62 K385H 0.50 K385N 0.41 K385Q 707 0.73 K385R 0.94 K385S 1.05 K385T 0.46 K385V 708 0.43 T387S 0.93 L388F 0.92 L388H 0.47 L388I 0.98 L388M 0.79 L388R 0.60 L388T 0.51 L388V 0.78 L388W 0.77 L388Y 1.18 E392W 0.31 E389A 709 1.14 E389G 710 0.91 E389H 1.17 E389K 712 1.91 E389L 711 0.65 E389M 0.60 E389P 0.75 E389Q 713 0.69 E389R 0.94 E389S 714 1.08 E389T 0.70 E389Y 0.77 L391C 0.90 E392A 715 0.58 E392F 716 0.54 E392G 1.00 E392K 0.66 E392L 0.80 E392M 717 1.54 E392Q 718 1.01 E392R 719 0.66 E392S 0.52 E392T 0.72 E392V 720 1.27 E392Y 0.92 Q393A 1.26 Q393D 0.45 Q393F 721 1.23 Q393H 1.05 Q393K 0.80 Q393L 0.91 Q393M 722 0.80 Q393N 0.72 Q393R 0.74 Q393S 1.15 Q393T 0.41 F394L 0.56 F394W 0.41 S395A 723 1.10 S395G 0.77 S395H 724 0.56 S395K 0.96 S395R 725 1.98 E396A 726 0.52 E396D 0.64 E396H 727 0.47 E396Q 728 0.73 E396R 0.61 E396S 729 0.61 E396T 0.89 E396L 0.39 Y399A 1.01 Y399C 0.46 Y399E 1.49 S407L 0.4 Y399K 730 1.94 Y399M 731 2.70 Y399N 0.52 Y399Q 1.18 Y399R 1.20 Y399S 1.01 Y399T 732 2.40 Y399V 733 1.44 Y399W 734 1.92 S401A 735 0.82 S401E 736 0.46 S401N 0.42 Y403F 0.62 S404A 737 0.63 S404P 0.64 S401G 0.38 T405F 0.36 T405A 0.56 T405G 738 2.32 T405K 0.74 T405M 0.48 T405P 0.64 T405Q 0.75 T405R 0.60 T405S 0.94 T405W 0.73

T405Y 0.44 L406A 0.70 L406C 0.98 L406E 0.73 L406F 739 1.42 L406G 1.00 L406I 0.61 L406N 740 0.76 L406Q 0.93 L406S 0.47 L406T 0.83 L406V 0.87 L406Y 0.74 S407A 741 1.16 S407D 742 1.52 S407E 743 1.38 S407F 744 1.42 S407G 0.75 S407H 745 1.34 S407M 0.74 K411H 0.33 S407N 0.72 S407P 747 0.94 S407Q 746 1.71 S407R 1.04 S407V 0.56 S407W 0.41 K409A 748 2.18 K409D 0.65 K409E 0.62 K409G 0.50 K409H 0.64 K409I 0.51 K409P 0.48 K409Q 749 3.33 K409R 0.84 K409S 0.72 I083K 0.30 K409T 0.63 K409V 0.48 A412Y 0.66 E410D 0.47 E410K 0.70 E410M 0.42 E410N 0.67 E410P 0.73 E410Q 0.85 E410R 0.61 E410S 0.81 E410T 750 1.54 E410V 0.65 E410Y 0.62 K411A 0.48 K411N 1.02 K411P 0.42 K411R 0.97 K411S 1.21 K411T 0.63 K411V 0.99 A412D 0.74 A412G 0.80 A412I 0.81 E220M 0.36 P226W 0.51 A412L 0.65 A412N 0.86 A412P 0.77 A412R 752 0.66 A412S 0.86 A412V 753 0.53 A412W 0.54 D413E 0.52 D413K 0.42 D413N 0.94 D413R 0.50 D413T 0.41 V414I 1.12 V414M 0.53 K415G 0.40 K415S 0.42 K415W 0.42 D416F 0.41 D416G 0.67 D416H 0.57 D416I 0.63 D416K 0.76 D416L 754 0.75 D416N 0.73 D416Q 0.83 D416R 0.46 V237C 483 0.35 D416T 0.85 D416V 0.59 D416Y 0.40 T417I 1.22 D413A 0.38 D413S 0.39 K415Y 0.39 D418A 0.92

D418E 755 1.31 D418F 0.81 L089P 0.38 D418G 0.45 D418I 0.99 D418L 756 1.28 D418M 1.09 D418N 0.91 D418P 757 2.11 D418Q 1.05 D418R 758 1.18 D418S 0.78 D418V 759 1.43 D418Y 0.97 A419E 0.45 A419F 760 2.17 A419G 0.42 A419H 761 1.21 A419I 762 1.64 A419K 763 1.88 A419L 0.56 A419N 0.53 V421I 0.39 A419R 764 1.81 A419S 765 2.65 A419W 0.69 A419Y 766 1.44 V420I 1.04 V420P 0.48 D421A 767 1.28 D421E 0.81 D421G 0.62 D421H 768 1.98 K255G 0.39 D421K 769 2.42 D421L 0.73 D421M 0.94 D421N 770 1.89 D421Q 771 1.54 D421R 772 2.21 D421S 773 2.12 K094C 0.33 D421T 0.80 D421Y 0.66 V422I 0.42 V422T 0.49 A425G 774 1.20 A425I 0.44 A425K 775 1.75 A425M 0.70 A425N 0.46 A425R 0.49 A425S 0.47 D426E 0.62 D426G 0.85 D426N 0.61 D426P 1.03 D426Q 0.42 D426Y 0.43 G427K 0.52 G427S 0.42 V428L 778 1.25 A425Y 0.39 G427T 777 0.35 G427Q 776 0.39 V428M 0.42 V428P 0.82 V428T 0.62 D431A 779 2.42 D431E 781 1.27 D431G 780 0.55 D431H 782 3.13 D431I 1.05 D431K 783 1.83 D431L 784 0.62 D431N 785 1.30 D431Q 786 2.16 D431R 787 2.20 D431S 788 1.91 D431V 789 1.52 D431W 0.56 D431Y 0.85 A432E 0.60 A432G 0.52 A432H 0.34 A432N 0.51 A432S 0.61 A432V 0.56 F433A 790 0.97 R270T 0.40 F433C 0.69 F433D 0.95 F433E 0.82 F433G 0.54 F433H 791 0.83 F433I 792 1.06 F433K 793 1.36 F433L 794 1.87 F433P 0.95 F433R 795 1.63 F433S 0.86 F433T 796 1.86 F433V 797 1.63 F433W 798 1.28 L434F 0.41 L434G 0.47 L263H 0.36 L434I 0.89 L434M 0.60 L434V 0.46 K435A 1.08 K435C 0.53 K435E 0.78 K435G 0.64 K435H 1.05 K435R 1.01 K435S 1.03 K435T 0.73 K435V 0.44 K435Y 0.50 P436D 1.19 P436E 0.74 P436G 1.19 L105H 0.36 P436H 0.72 V272M 0.31 P436I 0.84 P436K 799 2.05 P436L 0.63 P436M 0.61 P436Q 0.86 P436R 1.00 P436S 0.92 P436T 0.59 P436W 0.43 P436Y 0.49 P437A 0.56 P437D 0.62 P437G 0.50 P437H 1.11 P437I 800 2.46 P437K 0.83 P437L 0.51 P437M 801 2.55 P437Q 0.96 D275L 0.24 P437R 0.85 P437S 0.57 P437Y 0.42 M438A 802 0.75 M438C 0.63 M438D 803 0.87 M438E 804 0.72 M438G 0.83 M438L 805 0.86 M438N 806 1.08 M438P 0.81 M438Q 0.85 M438R 0.99 M438S 0.83 M438T 807 3.99 M438V 0.85 P125A 0.36 M438W 0.57 E439A 808 1.20 E439C 809 0.58 E439F 1.00 E439G 1.22 E439H 0.74 E439K 810 1.20 E439L 0.88 E439P 811 1.16 Q276G 0.36 E439Q 812 1.32 E439S 1.02 E439T 813 1.15 E439V 814 1.57 E439W 0.62 T440A 1.22 T440D 815 1.03 T440E 1.00 T440F 0.85 T440G 0.860.49 K279T 0.86 T440G 0.86 V128I 328 1.25 F280G 0.47 T440H 816 3.00 V128K 1.16 F280Q 0.43 T440I 1.04 V128L 0.95 S282D 0.41 T440L 0.97 V128Q 0.55 S282G 0.54 T440M 817 1.08 V128R 0.74 S282M 545 2.64 T440P 818 0.88 V128S 0.53 S282Q 0.41 T440R 819 1.77 V128W 0.50 Q283E 0.63 T440S 820 1.17 K130I 0.50 Q283P

1.18 T440V 1.02 K130R 329 1.42 Q283R 0.59 T440Y 1.11 N131C 0.60 Q283S 546 1.73 E441A 821 1.47 N131E 0.44 Q283T 0.65 E441D 0.67 N131F 0.63 D284A 0.58 E441F 822 3.91 N131G 330 2.47 D284E 1.21 E441G 0.87 N131H 0.80 D284G 0.60 E441H 0.65 N131I 331 1.40 D284H 0.51 E441K 0.80 N131L 0.82 D284L 0.50 E441L 0.82 N131M 332 0.99 D284M 0.56 E441N 0.82 N131Q 333 1.24 D284N 0.40 E441Q 0.81 N131R 334 2.81 D284Q 0.95 E441S 0.79 N131S 0.76 D284S 0.99 E441T 0.66 N131T 1.02 E285F 0.47 E441V 0.54 N131V 335 2.08 E285G 0.52 E441Y 0.51 N131Y 0.85 E285H 547 1.30 E442C 823 1.38 R132A 0.68 E285M 0.43 E442G 824 0.51 R132C 0.58 E285N 0.40 E442H 0.76 R132E 0.70 E285Q 0.59 E442K 0.73 R132F 0.60 E285Y 0.99 E442P 0.91 R132H 0.66 L286S 0.46 E442Q 0.74 K279A 0.27 D284T 0.39 D284Y 0.37 E285A 0.34 L286R 0.53 L286W 0.38 R132I 0.56 V287I 0.51 E442R 825 3.94 R132K 1.05 V287T 548 0.50 E442T 0.61 R132L 337 0.76 Y288L 0.79 E442V 0.65 R132N 336 1.28 Y288W 0.49 E442Y 0.60 R132Q 0.69 T289K 0.75 P443A 826 1.63 R132S 0.79 T289S 549 0.48 P443E 827 1.07 R132T 0.61 F290I 0.41 P443F 828 0.70 R132V 0.73 F290M 1.03 P443G 829 1.12 R132Y 0.78 G291Q 0.80 P443H 1.08 S133I 0.54 G291R 0.45 P443L 1.19 I134L 1.04 G291S 550 0.41 P443M 830 1.99 I134T 0.60 G291V 551 1.63 P443N 831 1.25 I134V 1.08 E292A 0.66 P443Q 0.96 E135A 0.99 E292C 552 0.71 P443R 1.04 E135C 0.77 E292F 553 0.90 P443S 0.99 E135D 338 2.68 E292G 0.41 P443T 0.87 E135F 0.73 E292H 554 1.26 P443W 0.64 E442L 0.4 E442W 0.38 Q444M 0.37 E135G 339 2.79 E292K 555 1.27 Q444D 0.97 E135H 0.79 E292N 0.99 Q444E 832 1.19 E135K 1.15 E292P 1.05 Q444F 0.66 E135L 0.82 E292R 556 0.42 Q444G 0.93 E135N 0.56 E292V 557 1.28 Q444H 833 0.97 E135Q 1.59 E292W 0.83 Q444I 0.58 E135R 340 2.08 T293A 558 1.90 Q444K 1.03 E135S 1.13 T293C 559 1.67 Q444N 1.01 E135W 0.63 T293D 560 1.46 Q444R 0.85 E135Y 0.50 V137C 0.37 Q444V 834 1.12 L136A 0.73 V137S 0.36 Q444W 0.64 L136C 0.56 V137L 0.21 Q444Y 0.67 L136D 0.47 Q143C 0.28 I445A 0.97 L136F 0.96 L144R 360 0.26 I445G 0.98 L136H 1.00 K152W 396 0.37 I445H 835 1.35 L136I 0.65 A153S 0.34 I445L 1.06 L136M 1.05 K154I 0.38 I445M 836 1.57 L136N 0.48 E156C 0.35 I445N 837 1.24 L136Q 0.61 E158G 0.37 I445P 838 1.67 L136R 0.74 K159G 0.38 I445Q 839 1.26 L136S 0.80 A160W 0.39 I445R 1.08 L136T 0.72 G161V 0.42 I445S 840 1.21 L136W 1.11 D163W 0.38 I445T 841 1.38 V137A 0.48 D163F 0.39 I445V 842 1.25 ~~I445W~~V137I 1.01 L165C 0.27 1445W 843 0.69 V137T 0.51 V166N 0.47 I445Y 0.53 Q138A 0.69 E167F 0.31 F446A 844 1.58 Q138C 0.65 K170A 0.40 F446C 0.75 Q138H 0.71 K170Q 0.40 F446D 1.18 Q138I 0.54 K173Q 0.32 F446E 1.10 Q138L 341 0.59 L174H 0.38 F446G 1.12 Q138M 0.68 R176L 0.40 F446H 1.28 Q138N 0.61 P177V 0.36 F446I 1.06 Q138R 0.53 L180I 0.38 F446K 0.94 Q138S 0.48 W181K 0.29 F446L 0.93 Q138W 0.41 Y183E 0.32 F446M 845 1.31 Q138Y 0.60 Y184W 0.39 F446Q 0.72 Q139A 0.92 H193R 0.33 F446R 0.89 Q139C 0.44 H193F 0.38 F446T 0.89 Q139D 0.48 K195V 0.36 F446V 0.91 Q139E 0.94 K196N 0.39 F446W 846 1.40 Q139F 0.53 K196Y 0.39 Y447D 847 3.25 Q139G 0.65 P197W 0.39 Y447E 848 1.36 Q139H 0.56 G198W 0.29 Y447F 1.41 Q139K 0.73 N200T 0.37 Y447G 849 0.92 Q139L 0.70 F204W 0.39 Y447I 850 1.36 Q139M 0.95 N205L 452 0.39 Y447L 1.09 Q139R 0.79 N205Y 0.4 Y447M 0.90 Q139S 0.81 V206Q 0.33 Y447N 851 1.58 Q139T 342 1.31 K209F 0.4 Y447P 852 1.46 Q139V 0.77 K209L 0.38 Y447Q 853 2.37 Q140A 0.96 N211L 0.41 Y447R 1.12 Q140C 0.50 N211W 0.51 Y447T 854 1.90 Q140D 0.59 W218M 0.38 Y447V 855 1.38 Q140F 0.66 W218V 0.28 Y447W 1.07

~~[0627]~~(530) 2. Inactive Mutants

~~[0628]~~(531) The other mutants that exhibited less than 20% hyaluronidase activity of wildtype PH20, in at least one of the duplicates, were rescreened to confirm that the dead mutants are

inactive. To confirm the inactive mutants, the hyaluronidase activity assay described in Example 3 was modified to incorporate an overnight 37~~.degree.~~° C. substrate-sample incubation step prior to measurement of enzymatic activity. The modified assay is intended to detect PH20 activities below 0.2 U/mL.

~~[0629]~~(532) The preparation of the bHA coated plates and blocking of the plates prior to addition of the transfected variant supernatants or wildtype PH20 was the same as described in Example 3. The assay was modified as follows. First, transfected variant supernatants or ~~wildtype~~wildypte PH20 not containing a mutation generated as described in Example 2 were diluted in duplicate 1:25 in assay diluent. For the standard curve, 1:3 serial dilutions of rHuPH20 (generated as described in Example 1) were made in assay diluent in duplicate starting from 0.1 U/mL down to 0.00014 U/mL. A blank well also was included. Then, 100 ~~.mu.l~~µl of the diluted samples or standard were added to pre-designated wells of the bHA-coated and blocked plate and allowed to incubate at 37~~.degree.~~° C. overnight. After the incubation, the plates were washed and binding to bHA detected as described above in Example 3. Optical density was measured at 450 nm within 30 minutes of adding the stop solution.

~~[0630]~~(533) The identified reconfirmed inactive mutants are set forth in Table 10. The Table sets forth the amino acid replacement compared to the sequence of amino acids of PH20 set forth

~~[0631]~~ in SEQ ID NO:3.

(534) TABLE-US-00010 TABLE 10 Inactive Mutants N002H R060V R121W C189P P236I V287N L336W G377V N002K R060Y R121Y C189R P236L V287P L336Y G378D N002W L061A N122A C189S P236N V287Q A337C G378E N002Y L061E N122C C189T P236Q V287R A337F G378F F003A L061F N122E C189V P236T V287S A337G G378I F003G L061G N122F C189W P236Y Y288D A337I G378L F003K L061H N122I C189Y A238F Y288E A337K G378M F003P L061N N122K Y190C A238G Y288F A337L G378Q F003T L061P N122Q Y190E A238L Y288G A337M G378T F003V L061Q N122R Y190F A238P Y288H A337R G378W R004D L061R N122S Y190G A238V Y288I A337T G378Y R004E L061T N122T Y190H A238W Y288K A337W K379A R004F L061W N122V Y190K A238Y Y288P A338C K379C R004G L061Y W123A Y190L A239C Y288R A338D K379E R004L G062A W123C Y190N A239F Y288T A338E K379F R004P G062C W123D Y190Q A239G T289A A338F K379I R004W G062D W123E Y190R A239H T289C A338G K379L R004Y G062F W123H Y190S A239I T289E A338H K379M A005D G062I W123L Y190T A239L T289G A338I K379W A005G G062K W123M Y190V A239P T289H A338K F380C A005I G062L W123P Y190W A239R T289L A338L F380D A005L G062M W123Q N191A A239S T289P A338P F380E A005M G062P W123R N191E A239T T289Q A338R F380G A005N G062Q W123S N191F A239V T289R A338T F380Q A005P G062R W123T N191G A239W T289S A338V F380R A005Q G062S W123V N191K A239Y T289Y K339D F380S A005R G062T W123Y N191L T240E F290D K339E T381G A005T G062V K124C N191M T240F F290Q K339F T381L A005V G062Y K124D N191P T240G F290Y K339G T381P A005W Y063C K124E N191Q T240N G291A K339H T381W A005Y Y063G K124F N191R T240W G291C K339L T381Y P006E Y063P K124N N191S T240Y G291D K339N V382E P006F Y064A P125C N191T L241A G291E K339P V382G P006T Y064C P125D N191V L241C G291F K339S V382H P006V Y064D P125G N191W L241D G291M K339T V382K P006Y Y064E P125L N191Y L241E G291N K339V V382L P007C Y064F P125N H192C L241G

G291T K339W V382M P007D Y064G P125W H192F L241I G291W K339Y V382N P007F Y064H K126F H192G L241P G291Y M340A V382P P007G Y064I K126H H192K L241R E292I M340C V382Q P007H Y064K K126I H192L L241S E292L M340D V382R P007I Y064L K126L H192M L241T E292T M340E V382S P007K Y064P K126N H192N L241V T293E M340F V382T P007L Y064Q K126P H192P L241W T293N M340G V382W P007Q Y064R K126Y H192Q Y242A V294A M340H V382Y P007R Y064S D127K H192R Y242C V294E M340K R383G P007S Y064T V128E H192V Y242D V294G M340P R383P P007T Y064V V128P H192W Y242G V294H M340R G384C P007W Y064W Y129A H192Y Y242I V294K M340S G384F P007Y P065A Y129C H193A Y242L V294L M340T G384M V008D P065C Y129D H193D Y242M V294N M340V G384Q V008E P065D Y129E H193K Y242P V294P M340W G384S V008G P065G Y129G H193L Y242R V294Q C341A G384T V008H P065H Y129H H193M Y242S V294R C341E K385C V008N P065I Y129L H193P Y242T V294S C341G K385L V008R P065K Y129P H193V Y242V V294T C341H K385M V008S P065N Y129Q Y194A Y242W V294W C341K K385P V008W P065R Y129S Y194C V243C A295C C341L K385W I009C P065S Y129T Y194I V243D A295G C341M K385Y I009D P065T Y129V Y194L V243F A295H C341N P386A I009E P065V Y129W Y194P V243G A295I C341Q P386C I009G P065W K130C Y194S V243H A295L C341R P386F I009N P065Y K130D Y194T V243L A295N C341S P386G I009P Y066A K130G Y194V V243M A295P C341T P386H P010F Y066C K130H K195S V243P A295T C341V P386I P010I Y066D K130L P197C V243Q A295V C341Y P386L P010L Y066E K130N G198V V243R A295Y S342D P386M P010M Y066G K130S G198W V243S L296C S342E P386N P010Y Y066I K130T Y199E V243W L296F S342F P386Q N011A Y066K K130W Y199G V243Y L296G S342H P386R N011C Y066L K130Y Y199H R244A L296I S342K P386S N011F Y066N N131P Y199I R244D L296K S342L P386T N011I Y066P R132P Y199K R244G L296M S342M P386V N011L Y066S S133D Y199L R244I L296Q S342P P386Y N011P Y066T S133E Y199P R244V L296R S342Q T387C N011T Y066V S133F Y199R R244Y L296S S342R T387E N011W I067D S133G Y199S N245A L296T S342T T387F N011Y I067E S133H Y199W N245C L296V S342Y T387G V012G I067G S133L N200A N245F L296W Q343C T387H V012H I067P S133M N200F N245L L296Y Q343D T387I V012W I067R S133N N200G N245P G297C Q343F T387L P013E I067T S133P N200H N245Q G297E Q343I T387M P013G I067W S133R N200K N245R G297H Q343P T387N P013I D068A S133T N200L N245S G297L Q343W T387V P013L D068C S133V N200M N245T G297N V344F T387W P013M D068G S133W N200P N245V G297P V344G T387Y P013V D068I I134A N200Q R246A G297Q V344H L388C F014A D068L I134C N200R R246C G297R V344L L388G F014E D068P I134D N200S R246D G297S V344M L388P F014G D068V I134F N200W R246E G297T V344N L388Q F014H D068Y I134G N200Y R246G G297Y V344P L388S F014K S069N I134H G201A R246H A298C V344Q E389F F014N S069T I134K G201F R246I A298E V344R E389V F014P I070Q I134P G201L R246K A298L V344S D390A F014Q T071P I134Q G201M R246L A298M V344T D390C F014W G072C I134R G201N R246M A298N V344W D390E L015E G072F I134S G201P R246P A298P V344Y D390F L015F G072H I134W G201R R246S A298Q L345A D390G L015G G072I E135P G201S R246T A298S L345C D390H L015K G072P L136P G201T R246V A298T L345E D390L L015N G072V V137F G201V R246W A298W L345H D390N L015P G072W V137G G201W V247A A298Y L345K D390P L015Q V073P V137H S202A V247C S299A L345N D390R L015R V075D V137N S202E V247F S299C L345Q D390S L015S V075G V137P S202F V247H S299D L345R D390T L015Y V075P V137R S202G V247N S299F L345T D390V W016A N076A V137W

S202H V247P S299G L345V D390W W016C N076C V137Y S202K V247Q S299H L345Y D390Y W016D N076F Q138V S202N V247R S299L C346A L391A W016E N076G Q139P S202P V247S S299M C346D L391D W016F N076I Q143C S202Q V247T S299P C346F L391G W016G N076K Q143H S202R V247W S299Q C346G L391H W016H N076L Q143P S202V V247Y S299T C346I L391K W016K N076P Q143R S202W R248C G300A C346K L391N W016M N076Q Q143S S202Y R248D G300C C346L L391P W016P N076R Q143T C203A R248E G300D C346M L391Q W016R N076S L144A C203D R248G G300E C346P L391R W016S N076T L144E C203E R248I G300F C346R L391S W016T N076V L144F C203G R248M G300L C346S L391T W016Y N076W L144I C203H R248P G300M C346T L391V A017D G077D L144K C203L R248T G300N C346V L391W A017E G077E L144P C203M E249A G300P C346W L391Y A017G G077L L144Q C203N E249G G300Q Q347C E392C A017H G077P L144S C203Q E249H G300S Q347F E392P A017I G077Q L144V C203R E249I G300T Q347I Q393C A017L G077R L144Y C203S E249K G300V Q347P Q393P A017N G077T S145T C203T E249M G300W Q347T F394A A017P G077V S145W C203V E249Q I301E Q347V F394D A017Q G078A A149E F204A E249S I301G Q347W F394E A017R G078D A149P F204C E249Y I301H E348C F394G A017S G078I T150V F204E A250C I301K E348H F394I A017T G078M K152L F204G A250F I301M E348I F394K A017V G078P A153E F204H A250G I301N E348L F394N A017W G078T A153F F204I A250H I301P E348P F394P A017Y G078Y A153M F204K A250K I301Q E348Q F394Q W018C I079A A153P F204Q A250L I301R E348R F394R W018D I079D A153R F204R A250M I301S E348T F394S W018F I079F A153T F204S A250N I301W E348V F394T W018G I079G A153V F204T A250P I301Y E348W F394V W018H I079H K154D V206C A250Q V302C E348Y S395C W018I I079K K154E V206D A250R V302D Q349D S395L W018L I079N K154G V206F A250S V302E Q349F S395M W018M I079P K154P V206G A250T V302F Q349G S395P W018P I079S K154S V206P A250V V302G Q349P E396C W018Q I079W K154W V206Y A250W V302H Q349V E396F W018S I079Y K154Y E207A I251D V302L Q349W E396G W018T P080A Q155P E207F I251F V302M Q349Y E396I W018V P080D Q155Y E207G I251G V302P G350A E396P W018Y P080E E156P E207M I251H V302R G350D E396Y N019A P080F F157A E207P I251K V302S G350E K397A N019C P080G F157C E207Q I251P V302T G350F K397C N019F P080I F157D E207R I251S V302Y G350H K397E N019G P080K F157E E207S I251T I303A G350K K397F N019H P080L F157G E207T I251W I303C G350L K397G N019I P080M F157H E207V R252A I303D G350M K397I N019L P080N F157I E207W R252D I303E G350N K397L N019M P080R F157K I208D R252E I303F G350P K397M N019P P080S F157L I208G R252F I303G G350R K397P N019Q P080T F157M I208P R252G I303K G350S K397Q N019R P080V F157P I208W R252H I303L G350T K397T N019S P080Y F157Q K209C R252I I303M G350V K397V N019V Q081A F157R K209P R252K I303R G350Y F398A N019W Q081C F157S R210A R252L I303W V351C F398C N019Y Q081E F157T R210C R252N I303Y V351D F398E A020D Q081G F157V R210D R252P W304A V351E F398G A020E Q081H E158D R210E R252S W304C V351F F398H A020F Q081L E158K R210G R252T W304D V351H F398I A020H Q081N E158P R210K R252Y W304G V351N F398L A020K Q081P E158R R210M V253A W304I V351R F398N A020L Q081S E158Y R210N V253D W304M V351W F398P A020N Q081V K159W R210P V253E W304N V351Y F398R A020P Q081W K159Y R210S V253G W304P C352A F398S A020R Q081Y G161W R210T V253H W304Q C352D F398T A020T K082W D163C R210V V253L W304S C352E F398V A020V K082Y D163P R210W V253M W304T C352F F398W A020Y I083E F164A R210Y V253N W304V C352G F398Y P021A I083K F164C

N211C V253Q W304Y C352K Y399D P021C S084Y F164D N211F V253R G305L C352M Y399P P021D L085A F164E N211G V253S G305P C352P C400A P021E L085C F164G N211H V253W G305Q C352Q C400D P021G L085D F164H N211I S254C G305R C352R C400E P021H L085E F164N N211K S254D G305S C352S C400F P021I L085F F164P N211M S254E G305T C352T C400G P021L L085G F164Q N211P S254G G305V C352V C400I P021M L085H F164R N211R S254I G305Y C352W C400L P021R L085N L165C N211S S254K T306A C352Y C400M P021S L085Q L165H N211T S254L T306C I353C C400P P021T L085S L165P N211V S254P T306H I353F C400Q P021V L085T L165T N211W S254Q T306I I353G C400R P021W Q086C V166D D212A S254R T306L I353H C400S S022C Q086P E167V D212G S254T T306V I353K C400T S022E D087P T168A D212H S254V T306W I353L C400V S022G H088A T168C D212I S254W T306Y I353M C400Y S022K H088C T168D D212K S254Y L307C I353Q S401C S022P H088E T168E D212L K255C I353R S401F E023A H088F T168F D212M K255D L307I I353S S401H E023F H088G T168G D212P K255L L307P I353W S401K E023L H088I T168K D212V K255P S308C R354C S401R E023M H088K T168L D212W K255V S308F R354D S401W E023N H088L T168P D213P K255W S308L R354E S401Y E023P H088M T168R D213S I256C S308M R354G C402A E023R H088P T168S L214A I256D S308V R354H C402D E023S H088R T168V L214C I256E S308W R354I C402E E023T H088S T168W L214D I256G S308Y R354K C402F E023V H088T T168Y L214E I256P M310C R354L C402L C025D H088V I169A L214G P257D M310E R354M C402M C025E H088Y I169D L214H D258L M310F R354P C402P C025F L089A I169F L214K D258P M310K R354Q C402Q C025G L089D I169G L214N D258V M310L R354S C402R C025H L089E I169H L214P D258W R311C R354V C402S C025I L089G I169K L214R K260C R311E R354W C402T C025K L089Q I169N L214S K260P R311F R354Y C402V C025L L089S I169P L214T S261P R311I K355D C402W C025N L089T I169Q L214Y P262A R311L K355F C402Y C025P L089W I169S S215C P262D R311P K355G Y403A C025R L089Y I169T S215P P262E R311V K355H Y403C C025S D090C I169Y W216D P262F R311W K355L Y403E C025T D090G K170C W216E P262G S312C K355M Y403G C025V K091D K170D W216G P262H S312E K355N Y403H C025Y K091E K170E W216H P262I S312M K355P Y403K G027C K091F K170G W216I P262K S312V K355Q Y403L L033C K091G K170M W216K P262Q S312W K355R Y403M L033D K091H K170P W216L P262R M313C K355S Y403N L033H K091I K170W W216M P262S K314C K355T Y403P L033N K091L K170Y W216N P262T K314L K355V Y403Q L033V K091N L171C W216P P262V K314W K355W Y403R L033Y K091T L171D W216Q P262W S315C K355Y Y403T D034I A092E L171H W216R P262Y S315I N356C S404C D034L A092F L171M W216T L263E S315V N356G S404D D034N A092H L171N W216V L263F C316E N356K S404F D034S A092K L171R L217A L263P C316G N356L S404G D034T A092P L171S L217C L263Q C316I N356P S404H D034V A092Q L171W L217G L263W C316K N356R S404L M035A A092R L171Y L217H P264D C316L N356T S404M M035D A092W G172D L217P P264E C316M N356V S404N M035G A092Y G172E L217Q P264F C316P N356W S404R M035P K094G G172I L217S P264G C316R W357D S404V M035R K094P G172L L217T P264L C316S W357E S404W M035S D095A G172P L217V P264M C316T W357F S404Y S036C D095C G172Q L217W P264R C316V W357G T405C S036F D095E G172T W218A P264T C316W W357L T405I S036V D095F G172V W218I P264V C316Y W357M T405V S036W D095G G172W W218K P264W L317G W357Q L406P S036Y D095H G172Y W218L P264Y L317P W357R L406R L037C D095K K173D W218P V265A L318C N358E C408A

L037E D095L K173E W218S V265D L318P N358H C408E L037G D095M K173G W218V V265F L318W N358I C408F L037N D095P K173H N219P V265G L319C N358K C408G L037S D095Q K173I E220G V265H L319E N358P C408I F038E D095S K173L E220K V265K L319F N358Q C408K F038G D095V K173M E220N V265L L319G N358R C408L F038K D095W K173P E220P V265M L319H N358W C408P F038L D095Y K173S E220R V265N L319I S359A C408R F038N I096A K173V E220W V265Q L319K S359F C408S F038Q I096C K173W S221D V265R L319M S359G C408T F038R I096G K173Y S221E V265S L319P S359L C408V F038T I096H L174P S221H F266A L319Q S359P C408W F038W I096P L175C S221K F266C L319R S359W C408Y S039C I096R L175D S221P F266G L319S S360A E410W S039D I096S L175G S221R F266H L319V S360C K411D S039F I096T L175K T222P F266M L319W S360E K411E S039W I096W L175P T222Y F266P L319Y S360F K411F F040A F098P L175R A223C F266Q D320C S360G K411G F040D Y099C L175S A223D F266R D320P S360I A412E F040E Y099E R176A A223E F266S D320V S360K A412H F040G Y099G R176C A223G F266T N321E S360L D413H F040K Y099I R176E A223H F266V N321M S360M D413I F040N Y099N R176F A223K F266W N321P S360P D413K F040R Y099P R176G A223L A267D Y322C S360Q D413L F040S Y099V R176H A223P A267G Y322D S360R D413P F040T Y099W R176I A223Q A267H Y322E S360V V414A F040V M100C R176P A223R A267I Y322G D361A V414D I041Q M100E R176Q A223S A267K Y322I D361C V414E G042D M100F R176S A223T A267N Y322L D361E V414G G042E M100G R176T A223V A267R Y322N D361G V414H G042H M100N R176V A223W A267S Y322P D361M V414K G042I M100P R176W A223Y A267W Y322R D361N V414R G042K M100R P177A L224A Y268A Y322S D361P V414S G042L M100S P177C L224D Y268C Y322T D361Q V414T G042M M100T P177D L224E Y268F Y322V D361R K415C G042P M100W P177F L224F Y268G Y322W D361S K415D G042Q M100Y P177G L224G Y268H M323A D361V K415E G042R P101A P177H L224M Y268K M323C D361W K415P G042S P101C P177L L224P Y268L M323E Y362A D416C G042T P101F P177M L224Q Y268N M323G Y362C D416S G042V P101H P177Q L224R Y268P M323H Y362E T417A S043A P101I P177R L224S Y268Q M323K Y362G T417D S043E P101K P177S L224T Y268S M323N Y362H T417E S043F P101L P177T L224W Y268T M323R Y362K T417F S043G P101M P177V L224Y Y268V M323S Y362L T417G S043I P101N P177W Y225A Y268W M323T Y362M T417H S043K P101Q N178E Y225D T269E M323V Y362N T417K S043L P101R N178I Y225E T269K E324C Y362P T417M S043Q P101S N178L Y225G T269L E324F Y362R T417P S043R P101T N178V Y225H T269M E324P Y362S T417Q S043V V102P N178W Y225K T269N E324V Y362T T417R P044A D103A N178Y Y225P T269P E324W Y362V A419D P044C D103E H179W Y225Q T269Q E324Y Y362W A419P P044F D103F L180A Y225R T269R T325C L363A V420A P044G D103G L180C Y225T R270A T325R L363C V420D P044H D103H L180E Y225V R270C I326E L363D V420F P044I D103I L180P Y225W R270E I326G L363E V420G P044L D103L L180R P226A R270F I326H L363F V420H P044N D103Q L180S P226C R270G I326N L363G V420K P044Q D103R W181A P226D R270H I326W L363H V420L P044R D103T W181C P226E R270I L327A L363I V420N P044S D103V W181D P226F R270P L327E L363P V420R P044T D103W W181E P226G R270Y L327F L363Q V420S P044W D103Y W181F P226L I271A L327G L363R V420T P044Y N104F W181H P226N I271D L327H L363S V420W R045A N104P W181I P226Q I271E L327N L363T V420Y R045D N104W W181K P226R I271H L327Q L363V V422C R045F L105C W181L P226S I271K L327R L363W V422D R045G L105M W181R P226T I271T L327S H364A V422G R045P L105N W181S P226V I271W L327T H364C

V422H R045W G106A W181V P226W V272A L327V H364D V422L I046P G106C G182A
P226Y V272H L327W H364E V422M I046W G106D G182C S227A V272L L327Y H364F
V422N N047V G106F G182D S227F V272N P329C H364G V422Q A048P G106H G182E
S227G V272P P329F H364K V422R T049C G106L G182H S227H V272W P329G H364L
V422S T049D G106M G182N S227I F273A P329H H364M V422Y T049G G106N G182P
S227K F273C P329I H364P C423A T049H G106P G182Q S227L F273D P329K H364R
C423D T049P G106S G182R S227M F273G P329L H364S C423E G106W G182S S227P
F273I P329N H364T C423F Q051C G106Y G182T S227Q F273L P329Q H364V C423G
Q051F M107A G182V S227R F273P P329R H364Y C423H Q051I M107C G182Y S227T
F273Q P329S L365A C423L Q051M M107H Y183C S227V F273S P329T L365C C423M
Q051P M107K Y183D S227W F273V P329V L365D C423P Q051T M107P Y183E S227Y
F273W P329W L365E C423Q Q051W M107Q Y183G I228A T274C P329Y L365G C423R
Q051Y M107S Y183I I228E T274E Y330A L365M C423S G052C M107V Y183K I228F
T274G Y330C L365N C423T G052E M107W Y183N I228G T274H Y330D L365P C423V
G052F A108D Y183P I228H T274N Y330E L365Q C423W G052W A108E Y183Q I228L
T274Q Y330G L365R I424A G052Y A108F Y183R I228M T274W Y330I L365S I424C
V053A A108K Y183S I228N T274Y Y330L L365T I424E V053C A108L Y183V I228P
D275A Y330M L365W I424G V053D A108M Y184A I228R D275F Y330N L365Y I424H
V053E A108P Y184C I228S D275G Y330P N366A I424N V053G A108Q Y184D I228T D275I
Y330R N366C I424Q V053H A108T Y184E I228W D275K Y330S N366E I424R V053L
A108V Y184F Y229E D275L Y330V N366F I424S V053N A108Y Y184G Y229F D275M
Y330W N366G I424W V053P V109C Y184H Y229G D275Q I331A N366K I424Y V053Q
V109D Y184K Y229K D275T I331C N366M A425E V053R V109E Y184L Y229L D275V
I331D N366P A425L V053S V109L Y184M Y229P D275W I331E N366Q A425P V053T
V109M Y184P Y229Q Q276F I331F N366R A425W V053W V109R Y184R Y229T Q276P
I331H N366T A425Y V053Y V109T Y184S Y229V Q276W I331K N366W D426C T054D
V109W Y184V Y229W L278M I331Q P367E D426F T054E I110F L185A L230A L278P
I331R P367F D426M T054G I110K L185D L230E K279A I331S P367I D426R T054P I110L
L185E L230G K279C I331T P367L G427A T054R I110M L185F L230H K279F I331W P367M
G427C T054Y I110P L185G L230K K279G I331Y P367Q G427F I055A I110W L185I L230M
K279L I332A P367V G427L I055D D111H L185K L230N K279W I332C D368C G427P
I055G D111I L185P L230P K279Y I332D D368P I055H D111Q L185R L230R F280D I332E
D368W G427V I055N W112C L185S L230S F280I I332F N369C G427W I055P W112E
L185T L230T F280L I332G N369E G427Y I055Q W112G L185V L230V F280M I332H
N369F V428A I055R W112H L185W L230W F280N I332K N369I V428C I055T W112L
L185Y L230Y F280R I332L N369K V428D I055V W112N F186A N231A F280S I332N
N369L V428E I055Y W112P F186D N231C F280T I332P N369P V428G F056A W112S
F186G N231D F280V I332R N369Q V428H F056C E113R F186H N231F F280W I332S
N369V V428N F056E E113V F186I N231G L281A I332T N369W V428R F056G E114I
F186K N231H L281D I332Y F370A V428S F056H E114L F186L N231I L281G N333G F370D
V428Y F056I E114P F186N N231K L281H N333H F370E C429A F056K E114T F186P
N231L L281I N333I F370G C429D F056L E114V F186Q N231P L281K N333K F370H
C429K F056P W115A F186R N231Q L281N N333P F370K C429L F056R W115C F186S
N231R L281P N333R F370L C429N F056S W115D F186V N231S L281Q N333S F370N
C429P F056T W115F F186W N231V L281R N333T F370P C429S F056V W115G P187A
T232C L281S N333W F370Q C429T F056W W115H P187F T232G L281V N333Y F370R

C429V Y057A W115I P187G T232H L281W V334A F370S C429W Y057D W115K P187H T232K S282F V334C F370V C429Y Y057F W115L P187I T232L S282L V334D F370Y I430A Y057G W115M P187L T232N S282V V334E A371P I430D Y057I W115R P187M T232P S282W V334G A371W I430E Y057L W115S P187N T232Q S282Y V334M I372A I430L Y057M W115V P187Q T232V Q283A V334N I372D I430M Y057P W115Y P187R T232Y Q283C V334R I372E I430N Y057Q R116A P187S Q233D Q283D V334S I372F I430S Y057R R116C P187T Q233I Q283F T335F I372G I430T Y057V R116D P187V Q233P Q283W T335G I372H I430V Y057W R116E P187W Q233S D284C T335H I372K D431P V058A R116G P187Y Q233T D284I T335I I372L A432C D059A R116H D188A Q234A D284P T335K I372N A432F D059E R116I D188C Q234D E285K T335L I372P A432I D059I R116L D188F Q234E E285P T335P I372R A432K D059L R116N D188G Q234G E285R T335V I372S A432L D059M R116P D188H Q234H E285T T335W I372T A432M D059P R116Q D188L Q234N E285V T335Y I372V A432P D059R R116S D188M Q234P L286A L336A I372W A432Y D059T R116V D188N Q234S L286C L336E Q373C L434H D059V R116W D188P Q234T L286D L336F Q373P L434K D059W P117D D188Q Q234V L286F L336G Q373W L434P D059Y P117G D188R Q234W L286H L336K L374D L434Q R060A P117I D188S S235F L286K L336N L374E L434R R060D P117K D188T S235L L286M L336P E375C L434W R060F P117N D188V S235M L286P L336R E375F P437T R060G P117Q D188W S235R L286T L336S E375P M438Y R060H P117R C189A S235W L286Y L336T E375V E439N R060I P117S C189E S235Y V287A L336V E375Y E439R R060L P117V C189G P236C V287C R121G K376I T440Q R060N P117W C189H W119L V287D R121H K376P E441R R060P T118C C189K W119N V287E R121K K376W E442M R060Q T118D C189L W119P V287G R121L G377C E442N R060S T118E C189M W119R V287K R121M G377I E442S R060T T118G C189N R121A V287L R121P G377L P443D T118R T118P T118W R121C R121F G378D G377V G378E T118Y W119I W119A W119K R121E G378F G378I

Example 5

Assay for Hyaluronidase Activity Under Temperature and Phenophilic Conditions

~~[0632]~~(535) Supernatants from PH20 activity variants set forth in Table 9, as identified in Example 4, were tested for stability under thermophilic and/or phenophilic conditions. The assay to measure hyaluronidase activity under temperature and phenophile conditions using biotinylated-HA (bHA) as substrate for measuring hyaluronidase activity was modified from the original assay described in Example 3 in that it incorporated a 4-hour 37~~.degree.~~° C. incubation of samples with or without m-cresol prior to measurement of enzymatic activity. The assay was used to identify PH20 mutants with thermophilic properties (activity greater at 37~~.degree.~~° C. condition than at 4~~.degree.~~° C.) and/or with ~~phenolphilic~~phenophilic properties (greater activity in the presence of m-cresol than wildtype PH20).

~~[0633]~~(536) 1. Primary Screen

~~[0634]~~(537) Prior to incubating samples with bHA, variant PH20 samples were diluted into designated wells of an uncoated ~~4.times.HB~~4XHB plate for pre-incubation at 37~~.degree.~~° C. for 4 hours under the following conditions: 1) pre-incubation at 37~~.degree.~~° C. with 0.4% m-cresol; and 2) pre-incubation at 37~~.degree.~~° C. without 0.4% m-cresol. For the preincubation at 37~~.degree.~~° C. with 0.4% m-cresol, a 1% m-cresol intermediate stock was prepared from 50%

(v/v) m-cresol stock solution. Briefly, in a 2 mL Wheaton glass vial a 50% stock of m-cresol (Fluka, Catalog No. 65996; Spectrum, Catalog No. C2773) was made in methanol based on the density (D=1.034 g/L). The vial was sealed and stored at ~~-20.degree.~~−20° C. with protection from light in small aliquotes. Then, the 1% intermediate stock was generated by dilution in HEPES assay buffer (10 mM HEPES, 50 mM NaCl, 1 mM CaCl.sub.2, 1 mg/mL BSA, pH 7.4, 0.05% Tween-20) daily immediately prior to use in a fume hood with vortexing.

~~[0635]~~(538) Then, duplicates of transfected variant supernatant samples set forth in Table 9, generated as described above in Example 2, were each separately subjected to a 1:2.5 dilution of 1% m-cresol in HEPES assay buffer/transfected supernatant to obtain 0.4% final concentration of m-cresol. For the preincubation at 37~~.degree.~~° C. without 0.4% m-cresol, transfected variant supernatant samples were subjected to a 1:2.5 dilution in HEPES assay buffer/transfected supernatant. In addition, for each condition, an internal killing control was also tested by spiking in 3 U/mL of rHuPH20 in pH 7.4 HEPES buffer (generated as described in Example 1) that was diluted the same as described above for the transfected samples. The plates were sealed with plate sealers and incubated at 37~~.degree.~~° C. for 4 hours.

~~[0636]~~(539) The preparation of the bHA coated plates and blocking of the plates prior to addition of the transfected variant supernatants or wildtype PH20 was the same as described in Example 3. The assay was further modified as follows. First, samples were diluted in duplicate 1:10 in HEPES assay buffer in ~~4.times.HB~~4XHB plates. For each variant, the samples that were tested were 1) non-preincubated transfected variant supernatant (no incubation; 4~~.degree.~~° C.); 2) preincubated transfected variant supernatants preincubated at 37~~.degree.~~° C. for 4 hours with 0.4% m-cresol (Cresol); or 3) preincubated transfected variant supernatant preincubated at 37~~.degree.~~° C. for 4 hours without 0.4% m-cresol (no cresol; 37~~.degree.~~° C.). In addition, the spiked-in samples also were tested. A standard curve using rHuPH20 was made as described in Example 3 without m-cresol. One hundred microliters (100 ~~.mu.l~~µl) of each standard and sample were transferred to pre-designated wells of the bHA-coated and blocked plate and incubated for approximately 1.5 hours at 37~~.degree.~~° C. Thus, each sample of each variant was tested in quadruplicate due to the preincubation of duplicate samples of each transfected variant supernatants in the pre-incubation step and the further duplicate of each sample in the bHA assay.

~~[0637]~~(540) After the incubation, the plates were washed and binding to bHA detected as described above in Example 3. Optical density was measured at 450 nm within 30 minutes of adding the stop solution.

~~[0638]~~(541) The U/mL activity was calculated from the standard curve and compared. The results were depicted as the percent (%) activity remaining under each of the following parameters: ratio of activity at 1) 37~~.degree.~~° C. preincubation without m-cresol/4~~.degree.~~° C.; 2) 37~~.degree.~~° C. after preincubation with m-cresol/4~~.degree.~~° C.; and 3) 37~~.degree.~~° C. after preincubation with m-cresol/after preincubation at 37~~.degree.~~° C. without m-cresol. Initial phenophile hits for reconfirmation were identified as those that in a duplicate assay exhibited a percentage of remaining activity under condition 3) of ~~.gtoreq.~~≥20% of the original activity at 37~~.degree.~~° C.

[0639](542) Initial Hits were rescreened using a 6-well plate rescreen assay. For the rescreen, plasmid DNA corresponding to the potential Hit was transformed into E. coli bacteria and plasmid DNA prepared and purified using MaxiPrep according to the manufacturers instructions. The DNA sequence was confirmed.

[0640](543) The plasmid DNA was transfected into monolayer CHO-SCHO—S cells (Invitrogen, Cat. No. 11619-012) grown on 6-well plates at a density of about 50-80% confluency using Lipofectamine 2000 (Invitrogen, Cat. No. 11668-027) according to the protocol suggested by the manufacturer. Transfections were performed in duplicate. The cells were incubated at 37.degree.° C. in a CO.sub.2 incubator for 96 hours post-transfection before collecting the supernatant for the assay. As controls, cells also were transfected with the HZ24-PH20(OHO)-IRES-SEAP expression vector (SEQ ID NO:4) that contains a codon-optimized wildtype PH20 sequence (OHO). Mock cells also were included as controls.

[0641](544) Ninety-Six (96) hours post-transfections, supernatant was collected from each sample, including the OHO and mock controls, and assayed for hyaluronidase activity under various conditions as described above: 1) non-preincubated transfected variant supernatant (no incubation; 4.degree.° C.); 2) preincubated transfected variant supernatants preincubated at 37.degree.° C. for 4 hours with 0.4% m-cresol (Cresol; 37.degree.° C.); or 3) preincubated transfected variant supernatant preincubated at 37.degree.° C. for 4 hours without 0.4% m-cresol (no cresol; 37.degree.° C.). Hyaluronidase activity was determined as described above using the bHA assay.

[0642](545) The results were assessed as described above. Absolute hyaluronidase activity (U/mL) was generated from the standard curve. In addition, percent activity was determined as a ratio of activity at 37.degree.° C./4.degree.° C., 37.degree.° C. plus m-cresol/4.degree.° C., and 37.degree.° C. plus m-cresol/37.degree.° C. The results are set forth in Tables 11 and 12 below.

(546) TABLE-US-00011 TABLE 11 Absolute Hyaluronidase Activity 37.degree.° C. with m-cresol Mutant No incubation 37.degree.(4° C.) 37° C. no cresol m-cresol (37.degree. C. Mutant (4.degree.° C.) (37.degree.° C.) plus m-cresol) L001A 2.993 2.511 3.529 3.214 0.287 0.295 L001E 2.669 2.539 2.862 3.179 0.376 0.341 L001G 0.348 0.583 0.596 0.676 0.055 0.031 L001Q 5.135 6.443 6.133 5.719 0.621 0.636 L001R 5.603 4.390 6.576 7.042 0.458 0.396 P006A 2.965 3.208 4.088 3.495 0.404 0.435 V008M 1.376 1.401 1.856 1.678 0.000 0.008 I009Q 0.447 0.381 0.469 0.476 0.031 0.030 P010G 0.747 0.564 0.820 0.688 0.123 0.114 P010H 0.473 0.485 0.624 0.548 0.000 0.000 N011S 0.862 0.962 1.313 1.263 0.094 0.064 V012E 11.019 5.519 5.312 5.528 0.753 0.934 V012I 2.804 3.844 3.610 6.566 0.106 0.090 V012K 1.691 1.963 2.479 2.243 0.330 0.321 F014V 0.144 0.165 0.222 0.242 0.003 0.000 L015M 0.902 1.073 1.026 0.901 0.017 0.017 A020S 1.494 2.205 2.822 2.620 0.413 0.397 S022T 3.035 3.788 3.375 3.273 0.684 0.748 L026M 1.482 1.226 2.027 1.704 0.224 0.178 K028R 0.944 0.845 1.043 0.925 0.112 0.095 F029R 1.195 1.511 1.848 1.839 0.140 0.140 F029S 3.019 3.615 3.566 3.521 0.250 0.283 F029T 1.451 1.712 1.839 2.065 0.220 0.212 P032C 0.370 0.419 0.476 0.534 0.006 0.040 L033G 0.566 0.700 0.686 0.627 0.001 0.026 D034W 0.340 0.321 0.499 0.471 0.076 0.069 M035V 0.887 0.639 0.721 0.652 0.116 0.023 S036H 1.109 0.752 1.178 1.135 0.117 0.026 S036N 0.797 0.933 0.893 0.859 0.171 0.260 L037M 0.574 0.404 0.455 0.353 0.049 0.032 F040L 2.603 3.941 3.515 4.148 0.277 0.361 I046L 3.027 2.959 4.011 3.342 0.513 0.557 N047D 2.222 2.359 2.573 2.639 0.032 0.021 N047W 0.404 0.415 0.423 0.456 0.000 0.017 A048N 12.398 45.971 14.252 23.873 0.797 0.902

T049R 7.893 13.334 9.685 12.102 0.563 0.649 G050D 3.287 3.148 3.084 3.020 0.242 0.264 G050M 1.763 2.333 2.780 3.244 0.250 0.393 G052N 7.217 9.809 6.939 13.978 1.109 1.083 G052T 1.542 1.224 1.795 1.433 0.381 0.463 G052S 2.152 1.999 2.120 1.963 0.498 0.566 V058C 1.428 1.312 1.321 1.301 0.212 0.210 V058K 28.000 28.000 61.016 61.016 23.586 23.586 V058R 5.719 4.688 5.542 4.822 3.134 3.149 V058N 1.200 1.175 1.550 1.525 0.200 0.175 V058Y 1.040 0.770 1.071 1.088 0.388 0.454 V058Q 11.956 15.363 18.458 45.092 1.567 2.166 V058P 3.360 2.949 2.799 5.121 0.592 0.884 V058H 3.790 5.074 7.590 9.222 0.826 1.205 D068P 0.215 0.215 0.213 0.180 0.001 0.184 S069T 1.927 2.179 2.671 2.671 0.289 0.240 I070P 1.284 1.593 1.306 1.589 0.010 0.032 I070V 1.818 2.437 3.099 3.335 0.433 0.363 V073Q 4.846 5.441 5.880 5.827 0.383 0.477 V073R 0.522 0.803 0.720 0.804 0.018 0.059 T074E 2.903 3.834 3.868 3.871 0.666 0.626 T074M 0.569 0.744 0.656 0.771 0.079 0.083 T074N 2.792 1.905 2.565 2.995 0.281 0.204 T074P 2.331 1.593 2.525 2.648 0.309 0.265 T074R 0.999 0.820 0.806 1.066 0.060 0.023 T074V 1.186 1.280 1.365 1.460 0.101 0.080 V075M 0.917 1.087 1.233 1.321 0.003 0.028 K082L 1.362 1.311 1.563 3.302 0.325 0.354 K082N 3.202 3.411 3.396 3.244 0.792 0.861 I083V 3.706 2.633 5.194 3.615 1.552 1.017 I083Q 2.376 1.946 2.665 3.674 0.720 0.510 I083S 0.841 1.054 0.880 1.005 0.235 0.268 I083G 2.276 2.443 2.418 1.866 0.545 0.601 S084E 1.470 1.484 1.834 1.683 0.115 0.115 S084F 1.179 1.212 0.982 1.103 0.025 0.000 S084N 2.255 1.888 3.268 2.476 0.597 0.547 S084R 8.534 14.779 10.230 30.016 1.117 1.494 Q086A 2.084 2.120 2.845 3.310 0.405 0.322 Q086H 1.187 1.000 1.218 1.296 0.087 0.065 Q086K 0.127 0.110 0.126 0.072 0.032 0.023 Q086S 2.528 2.082 2.539 2.149 0.173 0.241 Q086T 3.018 2.542 2.832 4.562 0.290 0.406 D087G 2.755 2.176 2.252 1.971 0.034 0.122 D087L 2.070 2.277 2.195 2.311 0.324 0.299 D087M 2.262 2.325 2.510 2.038 0.191 0.335 D087S 5.210 10.305 6.983 14.399 0.569 0.928 D087V 1.361 1.364 1.553 1.187 0.142 0.189 D090E 8.251 12.299 7.666 19.836 1.093 1.234 D090N 2.812 2.775 3.123 2.737 0.379 0.290 K093Q 2.491 2.065 2.267 1.971 0.132 0.131 K093R 2.986 2.862 3.094 2.842 0.362 0.465 K094D 2.393 2.088 2.071 2.132 0.135 0.211 K094R 1.407 1.542 1.764 1.676 0.158 0.166 T097C 0.330 0.618 0.545 0.505 0.044 0.087 T097D 0.520 0.565 0.643 0.664 0.055 0.073 T097E 1.096 1.410 1.394 1.623 0.217 0.262 T097L 0.899 1.198 1.065 1.241 0.246 0.300 N104R 2.508 2.356 2.876 2.790 0.279 0.238 A120H 2.155 2.551 2.028 2.883 0.168 0.199 D127R 0.264 0.339 0.149 0.199 0.105 0.068 V128I 3.120 3.313 3.546 3.401 0.389 0.504 N131M 15.335 20.678 27.143 15.899 0.505 0.447 N131R 8.195 8.748 7.724 8.392 1.645 1.626 N131V 1.656 1.870 2.280 1.962 0.233 0.214 R132L 3.306 3.235 3.259 2.966 0.337 0.430 Q138L 1.494 1.660 1.611 1.521 0.410 0.347 Q140K 2.829 4.065 4.996 4.464 0.546 0.559 N141R 1.290 1.320 1.334 1.527 0.058 0.035 N141S 2.201 2.708 2.900 2.966 0.135 0.164 N141W 1.475 1.568 1.927 1.643 0.100 0.105 V142D 2.552 2.186 2.914 3.193 0.128 0.067 V142G 1.357 1.796 1.597 1.621 0.211 0.219 V142K 3.532 2.381 3.867 3.681 0.571 0.575 V142N 0.432 0.567 0.672 0.589 0.103 0.087 V142P 4.624 7.213 7.722 7.021 1.074 1.081 V142Q 5.090 6.900 7.618 6.897 0.678 0.678 V142R 1.968 2.595 2.941 2.689 0.364 0.330 V142S 2.789 2.988 4.763 3.497 0.416 0.591 V142T 1.926 3.260 4.313 4.031 0.495 0.472 Q143G 3.922 4.903 5.632 4.846 0.782 0.780 Q143K 3.634 3.671 7.285 5.008 1.043 1.039 L144R 3.810 4.581 5.191 5.107 0.556 0.520 L144T 1.496 1.681 1.941 1.831 0.285 0.219 L146P 0.818 0.782 0.954 0.904 0.011 0.031 T147S 0.984 1.149 1.399 1.497 0.055 0.039 T150N 0.442 0.585 0.622 0.684 0.039 0.046 T150S 1.747 1.400 1.875 1.988 0.120 0.121 E151A 2.870 2.269 2.965 2.860 0.359 0.337 E151L 3.365 3.289 4.446 4.007 0.218 0.251 E151S 5.187 4.591 5.987 6.262 0.371 0.294 E151T 2.442 3.000 3.134 3.309 0.000 0.000 E151V 3.998 4.247 4.459 4.232 0.326 0.314 E151W 7.166 14.248 11.352 13.524 0.131 0.121 K152T 1.204 1.377 1.796 1.883 0.100 0.067 K152W 2.084 1.795 2.549 2.406 0.063 0.069 E158S 0.339 0.397 0.451 0.407 0.000

0.000 K162E 0.168 0.195 0.114 0.080 0.004 0.024 L165F 4.775 5.250 5.075 5.075 0.600 0.725 V166Q 1.883 2.507 2.937 2.958 0.392 0.324 V166T 0.993 1.315 1.821 1.800 0.231 0.235 E167D 0.811 0.910 1.109 1.480 0.111 0.056 I169L 1.812 1.796 2.540 2.196 0.335 0.341 K170R 1.578 2.054 2.536 1.995 0.209 0.201 G172A 0.413 0.581 0.692 0.777 0.052 0.056 K173R 1.654 1.551 1.766 2.083 0.173 0.156 L174G 0.184 0.087 0.210 0.230 0.026 0.031 L174N 1.616 2.276 2.494 2.872 0.331 0.543 L174T 0.552 0.566 0.689 0.820 0.090 0.050 N178K 2.931 4.375 4.891 4.513 0.258 0.362 N178R 8.160 13.820 16.287 20.033 0.665 0.790 H193Q 1.060 1.367 2.264 1.888 0.346 0.346 K195T 1.227 0.806 1.548 1.911 0.348 0.292 K195N 1.266 1.437 1.649 1.385 0.369 0.353 K196E 0.732 0.660 0.663 1.017 0.244 0.239 K196R 2.246 2.285 2.383 2.174 0.315 0.384 F204P 3.500 4.550 2.925 3.750 2.475 4.725 N205A 0.515 0.837 0.717 0.854 0.153 0.160 N205E 1.011 2.004 1.627 1.870 0.314 0.346 N205L 1.084 1.029 1.165 0.000 0.123 0.088 N205T 0.295 0.367 0.428 0.406 0.043 0.053 V206I 0.317 0.508 0.600 0.565 0.079 0.088 K209R 2.041 2.453 2.445 1.951 0.291 0.077 D212N 5.568 4.549 6.271 6.016 0.167 0.322 D212S 1.987 1.502 2.442 2.222 0.204 0.152 D213A 0.235 0.283 0.432 0.438 0.116 0.060 D213M 1.664 2.080 2.650 2.046 0.181 0.142 S215H 2.448 3.056 2.670 2.414 0.268 0.139 S215M 1.497 2.175 2.618 1.630 0.110 0.146 N219I 0.338 0.250 0.860 0.728 0.076 0.082 E220V 3.783 3.828 4.993 4.349 0.371 0.257 T222G 3.528 5.262 5.399 5.549 0.033 0.044 T232F 0.539 1.242 0.716 0.781 0.089 0.153 Q233G 0.041 0.095 0.115 0.121 0.000 0.000 Q234M 6.029 6.031 5.764 4.871 1.286 0.988 S235A 0.550 0.502 0.714 0.607 0.079 0.073 V237C 0.623 0.708 0.860 0.824 0.000 0.000 V237H 0.303 0.316 0.370 0.459 0.046 0.034 V237T 0.152 0.196 0.254 0.247 0.054 0.053 A238E 2.050 1.800 1.945 2.559 0.159 0.171 A238H 0.579 0.363 0.345 0.743 0.090 0.062 T240A 1.107 0.900 1.564 1.302 0.143 0.118 T240Q 0.333 0.510 0.542 0.617 0.080 0.085 R248A 2.274 2.499 2.575 3.115 0.027 0.075 E249V 3.001 3.894 4.284 4.325 0.655 0.712 P257G 3.981 4.452 4.985 5.022 0.039 0.034 K260M 0.719 0.960 0.839 0.935 0.072 0.068 S261A 3.253 3.117 1.872 2.686 1.264 1.451 S261K 6.089 5.421 9.860 6.297 1.583 1.437 S261N 14.149 40.257 20.219 14.303 2.115 1.917 A267T 0.052 0.095 0.102 0.106 0.036 0.041 F273H 0.340 0.436 0.417 0.519 0.025 0.031 F273Y 0.558 0.505 0.668 0.519 0.052 0.050 Q276H 2.706 1.877 2.027 1.997 0.181 0.201 Q276M 0.775 0.768 0.762 0.806 0.043 0.000 Q276R 6.080 9.717 7.383 14.593 0.807 1.281 Q276S 1.353 1.212 1.497 1.681 0.149 0.147 V277A 1.202 1.643 1.692 2.129 0.118 0.110 V277E 2.440 2.340 4.289 4.577 0.161 0.239 V277H 5.548 5.302 7.181 7.300 0.227 0.512 V277K 8.950 8.996 33.627 33.627 4.442 4.045 V277M 1.279 1.622 1.754 1.818 0.264 0.270 V277N 14.351 4.306 12.865 11.772 0.938 0.796 V277Q 5.459 5.461 6.547 6.343 0.373 0.493 V277R 18.300 12.038 17.581 20.641 2.737 2.023 V277S 14.351 10.444 9.509 15.135 0.727 0.716 V277T 8.412 7.804 8.497 11.184 0.679 0.871 L278E 4.416 2.795 3.330 2.800 0.170 0.202 L278G 7.502 7.456 9.173 7.760 0.596 0.612 K279H 0.888 1.087 1.234 1.339 0.185 0.269 V287T 0.580 0.667 0.843 0.832 0.139 0.100 T289S 0.783 1.019 0.819 1.001 0.008 0.007 G291S 0.227 0.322 0.419 0.385 0.051 0.016 G291V 3.662 3.707 4.131 5.599 0.821 0.706 E292C 1.344 1.599 1.711 1.617 0.138 0.144 E292F 6.106 4.697 8.422 6.216 0.520 0.363 E292H 2.620 3.316 4.458 3.830 0.389 0.451 E292R 2.810 2.178 3.155 2.829 0.398 0.339 E292V 0.891 1.121 1.453 1.494 0.193 0.177 T293A 1.986 3.110 2.546 1.789 0.086 0.076 A298G 0.161 0.274 0.342 0.236 0.030 0.022 L307G 0.616 0.661 0.726 0.605 0.000 0.000 S308D 0.264 0.325 0.337 0.344 0.014 0.010 S308K 0.651 0.722 0.826 0.716 0.011 0.000 S308N 3.995 4.406 6.808 6.128 0.386 0.362 I309E 3.166 2.819 3.921 3.663 0.637 0.528 I309G 6.651 5.429 6.824 6.194 0.503 0.400 I309L 0.326 0.403 0.501 0.431 0.048 0.047 I309M 2.809 2.473 3.467 3.383 0.278 0.239 I309N 4.865 5.191 5.444 5.054 0.380 0.327 I309S 10.719 28.759 18.217 158.604 0.748 1.367 I309T 3.052 2.509 2.989 3.735 0.228 0.207 I309V 1.705 1.292 1.929 1.787 0.029 0.062 M310G

4.514 6.397 7.568 7.084 0.866 0.915 M310Q 3.648 3.179 3.912 3.380 1.088 0.955 M313G 0.252 0.325 0.348 0.355 0.034 0.036 M313H 3.767 5.276 10.243 10.395 0.380 0.404 M313K 12.689 12.122 15.085 12.984 0.129 0.072 M313P 4.050 2.951 4.198 3.919 0.209 0.177 M313R 4.634 10.863 7.288 3.568 0.337 0.296 M313T 2.903 4.474 4.705 4.467 0.331 0.313 M313Y 1.063 1.262 1.276 1.300 0.096 0.089 K314S 2.848 4.450 4.042 5.879 0.391 0.533 K314Y 0.093 0.131 0.226 0.182 0.013 0.020 S315A 1.472 1.082 1.345 1.484 0.222 0.148 S315H 2.412 3.242 3.648 3.414 0.440 0.371 S315Y 0.279 0.626 0.477 0.362 0.146 0.143 L317A 3.254 2.845 4.019 3.776 0.280 0.317 L317I 1.078 1.524 2.021 1.687 0.257 0.180

L317K 12.129 9.382 11.668 12.591 0.402 0.445 L317N 2.907 3.066 3.703 3.717 0.445 0.540 L317R 8.631 15.187 20.585 15.106 0.796 0.857 L317S 11.586 29.267 10.535 25.114 1.637 1.613 L317T 1.338 1.073 1.953 1.656 0.136 0.018 L317W 0.810 1.128 1.326 1.665 0.158 0.171 L318D 1.750 1.970 1.847 1.930 0.322 0.322 L318H 1.073 0.806 1.072 1.005 0.046 0.074 L318R 2.856 3.464 4.583 4.187 0.258 0.260 N321R 3.069 4.409 5.059 4.946 0.482 0.426 N321S 0.683 0.710 0.700 0.772 0.058 0.035 E324N 4.309 2.530 4.508 3.321 0.348 0.303 T325E 1.071 1.270 1.337 1.352 0.193 0.143 N328G 0.379 0.504 0.747 0.553 0.031 0.040 N328Y 2.629 4.543 4.758 4.543 0.490 0.477 T335S 0.905 0.787 0.977 0.986 0.113 0.062 Q347A 8.316 11.961 8.432 11.508 0.918 1.266 Q347G 1.358 1.120 3.021 2.319 0.253 0.209 Q349M 1.493 1.629 1.486 1.760 0.178 0.217 Q349R 0.451 0.572 0.663 0.598 0.078 0.079 V351S 1.379 1.633 1.804 1.647 0.000 0.000 I353V 2.335 1.954 3.090 2.697 0.323 0.321 N356H 0.445 0.451 0.445 0.588 0.038 0.023 N356S 0.262 0.253 0.136 0.318 0.000 0.008 S359E 2.616 2.635 3.547 3.560 0.382 0.333 S359H 0.403 0.371 0.445 0.374 0.000 0.000 P367A 0.643 0.782 1.074 0.996 0.139 0.131 P367G 0.593 0.530 0.686 0.650 0.000 0.000 P367K 0.707 0.767 0.890 0.513 0.045 0.052 P367S 3.967 3.478 2.946 3.073 0.424 0.505 D368A 1.762 2.321 2.143 1.895 0.031 0.040 D368E 3.464 4.944 5.772 4.842 0.530 0.555 D368L 0.557 0.566 0.607 0.619 0.000 0.006 D368M 0.861 1.065 1.031 1.104 0.028 0.028 D368R 4.503 5.270 7.418 6.226 0.754 0.735 D368T 2.345 1.993 2.512 2.525 0.072 0.085 N369R 1.548 2.719 2.503 2.022 0.160 0.125 A371F 2.760 5.207 4.974 3.980 0.308 0.222 A371H 8.101 86.587 77.531 77.531 1.403 1.316 A371H 3.509 4.058 3.900 3.879 0.000 0.334 A371K 2.903 3.546 3.963 4.055 0.509 0.505 A371L 11.018 40.668 76.587 43.516 1.159 0.964 A371L 3.328 3.445 3.472 2.075 0.000 0.025 A371R 25.855 25.855 n/a n/a 2.851 3.634 A371R 6.592 7.733 7.987 7.576 0.000 0.196 A371S 3.329 3.505 4.916 4.611 0.412 0.781 L374P 2.939 7.129 11.522 8.771 0.665 0.646 E375A 0.627 0.507 0.557 0.683 0.000 0.014 E375G 1.596 1.299 2.025 1.806 0.209 0.265 E375R 0.937 1.132 1.529 1.318 0.201 0.260 K376D 0.458 0.312 0.518 0.515 0.064 0.026 K376E 1.572 1.094 1.572 1.674 0.213 0.174 K376Q 0.727 0.940 0.910 0.846 0.116 0.102 K376R 2.086 1.351 1.704 2.690 0.539 0.279 K376T 0.847 1.001 1.026 1.135 0.153 0.064 K376V 0.834 0.861 1.036 1.021 0.033 0.026 K376Y 1.316 0.777 1.353 0.747 0.125 0.097 G377D 1.159 1.332 1.285 1.763 0.202 0.186 G377E 0.877 0.926 1.144 1.189 0.092 0.088 G377H 3.037 3.432 4.460 3.598 0.372 0.364 G377K 3.445 4.101 6.405 4.911 0.283 0.245 G377R 1.096 1.257 1.312 1.191 0.077 0.085 G377S 0.453 0.452 0.492 0.457 0.034 0.036 G377T 2.198 2.313 2.474 2.522 0.424 0.461 F380W 17.497 27.987 25.734 29.353 2.566 2.716 T381S 2.861 3.161 3.886 3.558 0.521 0.367 R383I 1.959 6.936 10.340 6.820 0.655 0.513 R383S 2.429 2.548 3.228 3.044 0.339 0.321 K385A 0.479 0.669 0.604 0.754 0.028 0.000 K385Q 1.746 2.089 2.403 2.609 0.217 0.196 K385V 1.232 1.750 1.387 1.410 0.071 0.042 E389A 6.872 10.944 21.081 24.610 0.449 0.449 E389G 0.166 0.203 0.188 0.284 0.004 0.000 E389L 1.814 2.142 2.598 2.403 0.370 0.303 E389Q 2.547 3.432 3.459 3.423 0.411 0.437 E389S 1.847 2.640 3.059 2.456 0.000 0.007 E392A 1.797 1.370 2.021 2.133 0.147 0.136 E392F 1.575 1.407 1.821 2.023 0.071 0.079 E392Q 5.826 4.653 6.583 4.364 0.693 0.729 E392R 4.555 5.306 5.900 6.548

0.218 0.193 E392V 3.817 2.936 4.747 4.544 0.367 0.291 Q393F 1.754 2.186 2.455 2.222 0.260 0.226 Q393M 1.252 1.826 1.749 1.588 0.028 0.049 S395A 4.220 6.127 8.788 6.906 1.141 0.856 S395H 1.609 2.261 2.574 2.564 0.323 0.268 E396A 1.135 1.184 1.497 1.524 0.126 0.149 E396H 0.357 0.532 0.751 0.684 0.069 0.022 E396Q 1.310 1.625 1.611 1.559 0.162 0.160 E396S 3.375 5.709 5.274 6.380 0.146 0.129 Y399T 2.538 3.250 3.313 3.989 0.000 0.002 Y399V 2.738 2.697 3.028 3.129 0.484 0.557 Y399W 1.400 1.883 1.715 1.946 0.236 0.233 S401A 2.636 3.171 3.216 3.148 0.447 0.410 S401E 1.685 1.601 2.110 2.060 0.344 0.309 S404A 1.288 1.635 1.924 1.724 0.000 0.019 L406F 0.706 0.490 0.867 0.716 0.000 0.000 L406N 0.617 0.795 0.943 1.044 0.060 0.070 S407A 2.428 2.949 3.432 3.255 0.389 0.548 S407D 2.090 5.790 5.038 5.682 0.569 0.575 S407P 2.660 2.708 3.812 3.301 0.261 0.366 A412Q 2.001 2.918 2.925 2.902 0.279 0.247 A412R 4.562 5.132 6.390 6.347 0.570 0.596 A412V 2.581 3.451 3.789 3.511 0.189 0.189 D416L 0.610 0.817 0.737 1.043 0.130 0.160 D418R 4.541 4.847 5.347 5.438 0.406 0.583 A419H 10.409 20.311 25.109 38.221 2.214 2.293 A419K 12.835 10.298 24.536 208.289 2.556 3.173 D421A 5.968 5.617 6.094 16.940 0.761 0.764 D421H 48.012 48.012 160.106 32.481 16.300 28.113 D421K 5.527 5.225 6.864 5.346 0.523 0.725 D421N 9.060 8.635 10.039 8.645 1.502 1.422 D421Q 7.529 5.581 7.858 8.016 0.842 0.994 D421R 6.637 5.463 9.211 7.537 0.815 0.737 D421S 5.556 5.355 7.899 8.898 0.869 0.762 A425G 10.421 8.827 7.796 10.676 0.827 1.189 G427Q 1.008 1.252 1.342 1.230 0.031 0.106 G427T 1.330 1.380 1.664 1.643 0.080 0.065 V428L 2.138 2.769 2.930 3.029 0.053 0.030 D431E 2.810 2.220 1.972 2.112 0.519 0.438 D431H 2.154 3.185 4.017 3.028 0.294 0.301 D431K 8.123 16.953 19.563 11.575 2.272 2.339 D431L 1.211 1.215 1.564 1.448 0.164 0.170 D431N 11.819 12.063 16.358 15.131 1.601 1.399 D431Q 6.077 9.828 14.157 10.760 1.533 1.153 D431S 14.523 10.220 11.338 9.075 0.853 0.829 F433A 4.035 4.673 5.943 4.649 0.581 0.595 F433H 1.836 2.397 2.574 2.108 0.347 0.356 F433I 2.754 2.643 2.990 2.299 0.338 0.382 F433K 17.815 14.495 16.240 49.615 1.806 1.790 F433R 8.198 6.719 10.572 8.960 1.113 0.857 F433T 6.005 5.941 9.716 8.019 1.327 1.542 F433V 10.645 7.762 150.315 8.696 2.415 1.505 F433W 0.526 0.795 0.784 0.903 0.082 0.068 P437I 0.759 0.996 1.130 1.066 0.027 0.019 M438A 1.996 1.518 2.125 2.060 0.214 0.210 M438D 2.849 2.522 3.002 2.857 0.305 0.074 M438E 4.681 4.992 5.386 5.680 0.431 0.518 M438L 10.127 5.268 6.663 11.324 0.670 0.739 M438N 6.172 5.531 8.050 5.568 0.649 0.662 M438T 2.218 2.411 2.308 2.500 0.309 0.304 E439A 3.557 4.432 4.883 4.235 0.568 0.596 E439A 1.099 0.998 1.694 1.470 0.080 0.109 E439C 0.148 0.256 0.286 0.286 0.042 0.045 E439K 0.466 0.588 0.580 0.616 0.077 0.065 E439P 2.868 3.736 3.394 3.267 0.529 0.490 E439Q 1.070 0.848 1.087 1.080 0.116 0.115 E439T 1.965 1.889 2.179 2.323 0.313 0.263 T440D 4.148 4.443 4.931 3.533 0.568 0.651 T440H 2.317 1.982 3.297 2.595 0.147 0.196 T440M 3.397 3.305 2.878 2.873 0.254 0.367 T440P 3.562 3.593 3.987 3.277 0.540 0.566 T440S 2.522 2.207 2.533 2.895 0.283 0.284 E441F 1.402 1.407 1.813 1.560 0.204 0.178 E442G 2.871 3.340 3.193 3.347 0.327 0.367 P443E 0.907 0.710 0.856 0.928 0.044 0.063 P443F 1.830 2.370 2.683 2.321 0.301 0.286 P443G 4.077 2.921 9.751 4.614 0.835 0.756 Q444E 8.293 3.861 6.800 6.213 0.581 0.594 Q444H 3.823 3.936 5.746 4.710 0.486 0.513 Q444V 2.193 2.107 2.847 2.583 0.384 0.284 I445M 5.265 4.438 4.480 4.489 0.773 0.691 I445N 3.375 4.024 3.592 3.515 0.499 0.455 I445W 2.289 2.694 2.683 2.695 0.314 0.296 Y447E 2.373 2.464 2.363 2.685 0.391 0.345 Y447G 0.945 1.352 1.358 1.401 0.187 0.162 Y447P 0.991 1.383 1.379 1.490 0.190 0.183 positive 2.919 2.173 2.773 2.105 0.145 0.178 control 3.984 4.463 4.215 4.823 0.189 0.253 (OHO) 3 2.725 3 3.325 0.1 0.125 2.501 2.883 2.370 3.158 0.452 0.522 7.629 2.989 10.835 3.914 0.485 0.219 5.783 5.356 2.609 3.643 0.542 0.402 5.279 5.422 2.815 4.026 0.618 0.401 4.775 4.385 2.845 3.327 0.718 0.540 3.617 4.264 3.322 3.427 0.633 0.479 5.881 4.511 5.518 4.359 0.743 0.848 6.754 4.932 3.902 4.120 0.665 0.724

3.911 3.494 3.911 5.179 0.726 0.841 5.406 7.559 4.018 4.620 0.735 0.429 4.015 3.887 3.9400 3.4080 0.3340 0.3410 2.604 2.339 2.4430 2.3910 0.2350 0.2330 3.736 3.473 3.6210 3.0560 0.3100 0.2770 3.759 3.509 3.6330 3.0490 0.3600 0.3030 n/a (not available; e.g.~~,~~ beyond detection limit)

(547) TABLE-US-00012 TABLE 12 Percent (%) Activity duplicate 1 duplicate 2 % activity % activity % activity % activity % activity at 37~~.degree.~~° C. + m- 37~~.degree.~~° C. + m- at 37~~.degree.~~° C. + m- 37~~.degree.~~° C. + m- 37~~.degree.~~° C./4~~.degree.~~° C. cresol/37~~.degree.~~° C. cresol/4~~.degree.~~° C. 37~~.degree.~~° C./4~~.degree.~~° C. cresol/37~~.degree.~~° C. cresol/4~~.degree.~~° C. L001A 117.908 8.13 9.59 127.997 9.179 11.75 L001E 107.231 13.14 14.09 125.207 10.727 13.43 L001G 171.264 9.23 15.80 115.952 4.586 5.32 L001Q 119.435 10.13 12.09 88.763 11.121 9.87 L001R 117.366 6.96 8.17 160.410 5.623 9.02 P006A 137.875 9.88 13.63 108.946 12.446 13.56 V008M 134.884 0.00 0.00 119.772 0.477 0.57 I009Q 104.922 6.61 6.94 124.934 6.303 7.87 P010G 109.772 15.00 16.47 121.986 16.570 20.21 P010H 131.924 0.00 0.00 112.990 0.000 0.00 N011S 152.320 7.16 10.90 131.289 5.067 6.65 V012E 48.208 14.18 6.83 100.163 16.896 16.92 V012I 128.745 2.94 3.78 170.812 1.371 2.34 V012K 146.600 13.31 19.52 114.264 14.311 16.35 F014V 154.167 1.35 2.08 146.667 0.000 0.00 L015M 113.747 1.66 1.88 83.970 1.887 1.58 A020S 188.889 14.64 27.64 118.821 15.153 18.00 S022T 111.203 20.27 22.54 86.404 22.854 19.75 L026M 136.775 11.05 15.11 138.989 10.446 14.52 K028R 110.487 10.74 11.86 109.467 10.270 11.24 F029R 154.644 7.58 11.72 121.707 7.613 9.27 F029S 118.119 7.01 8.28 97.400 8.037 7.83 F029T 126.740 11.96 15.16 120.619 10.266 12.38 P032C 128.649 1.26 1.62 127.446 7.491 9.55 L033G 121.201 0.15 0.18 89.571 4.147 3.71 D034W 146.765 15.23 22.35 146.729 14.650 21.50 M035V 81.285 16.09 13.08 102.034 3.528 3.60 S036H 106.222 9.93 10.55 150.931 2.291 3.46 S036N 112.045 19.15 21.46 92.069 30.268 27.87 L037M 79.268 10.77 8.54 87.376 9.065 7.92 F040L 135.036 7.88 10.64 105.252 8.703 9.16 I046L 132.507 12.79 16.95 112.944 16.667 18.82 N047D 115.797 1.24 1.44 111.869 0.796 0.89 N047W 104.703 0.00 0.00 109.880 3.728 4.10 A048N 114.954 5.59 6.43 51.931 3.778 1.96 T049R 122.704 5.81 7.13 90.760 5.363 4.87 G050D 93.824 7.85 7.36 95.934 8.742 8.39 G050M 157.686 8.99 14.18 139.048 12.115 16.85 G052N 96.148 15.98 15.37 142.502 7.748 11.04 G052T 116.407 21.23 24.71 117.075 32.310 37.83 G052S 98.513 23.49 23.14 98.199 28.833 28.31 V058C 92.507 16.05 14.85 99.162 16.141 16.01 V058K 217.914 38.66 84.24 217.914 38.655 84.24 V058R 96.905 56.55 54.80 102.858 65.305 67.17 V058N 129.167 12.90 16.67 129.787 11.475 14.89 V058Y 102.981 36.23 37.31 141.299 41.728 58.96 V058Q 154.383 8.49 13.11 293.510 4.804 14.10 V058P 83.304 21.15 17.62 173.652 17.262 29.98 V058H 200.264 10.88 21.79 181.750 13.067 23.75 D068P 99.070 0.47 0.47 83.721 102.222 85.58 S069T 138.609 10.82 15.00 122.579 8.985 11.01 I070P 101.713 0.77 0.78 99.749 2.014 2.01 I070V 170.462 13.97 23.82 136.849 10.885 14.90 V073Q 121.337 6.51 7.90 107.094 8.186 8.77 V073R 137.931 2.50 3.45 100.125 7.338 7.35 T074E 133.241 17.22 22.94 100.965 16.172 16.33 T074M 115.290 12.04 13.88 103.629 10.765 11.16 T074N 91.870 10.96 10.06 157.218 6.811 10.71 T074P 108.323 12.24 13.26 166.227 10.008 16.64 T074R 80.681 7.44 6.01 130.000 2.158 2.80 T074V 115.093 7.40 8.52 114.063 5.479 6.25 V075M 134.460 0.24 0.33 121.527 2.120 2.58 K082L 114.758 20.79 23.86 251.869 10.721 27.00 K082N 106.059 23.32 24.73 95.104 26.541 25.24 I083V 140.151 29.88 41.88 137.296 28.133 38.63 I083Q 112.163 27.02 30.30 188.798 13.881 26.21 I083S 104.637 26.70 27.94 95.351 26.667 25.43 I083G 106.239 22.54 23.95 76.381 32.208 24.60 S084E 124.762 6.27 7.82 113.410 6.833 7.75 S084F 83.291 2.55 2.12 91.007 0.000 0.00 S084N 144.922 18.27 26.47 131.144 22.092 28.97 S084R 119.873 10.92 13.09 203.099 4.977 10.11 Q086A 136.516 14.24 19.43 156.132 9.728 15.19 Q086H 102.612

7.14 7.33 129.600 5.015 6.50 Q086K 99.213 25.40 25.20 65.455 31.944 20.91 Q086S 100.435 6.81 6.84 103.218 11.215 11.58 Q086T 93.837 10.24 9.61 179.465 8.900 15.97 D087G 81.742 1.51 1.23 90.579 6.190 5.61 D087L 106.039 14.76 15.65 101.493 12.938 13.13 D087M 110.964 7.61 8.44 87.656 16.438 14.41 D087S 134.031 8.15 10.92 139.728 6.445 9.01 D087V 114.107 9.14 10.43 87.023 15.922 13.86 D090E 92.910 14.26 13.25 161.281 6.221 10.03 D090N 111.060 12.14 13.48 98.631 10.596 10.45 K093Q 91.008 5.82 5.30 95.448 6.646 6.34 K093R 103.617 11.70 12.12 99.301 16.362 16.25 K094D 86.544 6.52 5.64 102.107 9.897 10.11 K094R 125.373 8.96 11.23 108.690 9.905 10.77 T097C 165.152 8.07 13.33 81.715 17.228 14.08 T097D 123.654 8.55 10.58 117.522 10.994 12.92 T097E 127.190 15.57 19.80 115.106 16.143 18.58 T097L 118.465 23.10 27.36 103.589 24.174 25.04 N104R 114.673 9.70 11.12 118.421 8.530 10.10 A120H 94.107 8.28 7.80 113.015 6.903 7.80 D127R 56.439 70.47 39.77 58.702 34.171 20.06 V128I 113.654 10.97 12.47 102.656 14.819 15.21 N131M 177.000 1.86 3.29 76.888 2.811 2.16 N131R 94.253 21.30 20.07 95.930 19.376 18.59 N131V 137.681 10.22 14.07 104.920 10.907 11.44 R132L 98.578 10.34 10.19 91.685 14.498 13.29 Q138L 107.831 25.45 27.44 91.627 22.814 20.90 Q140K 176.600 10.93 19.30 109.815 12.522 13.75 N141R 103.411 4.35 4.50 115.682 2.292 2.65 N141S 131.758 4.66 6.13 109.527 5.529 6.06 N141W 130.644 5.19 6.78 104.783 6.391 6.70 V142D 114.185 4.39 5.02 146.066 2.098 3.06 V142G 117.686 13.21 15.55 90.256 13.510 12.19 V142K 109.485 14.77 16.17 154.599 15.621 24.15 V142N 155.556 15.33 23.84 103.880 14.771 15.34 V142P 166.998 13.91 23.23 97.338 15.397 14.99 V142Q 149.666 8.90 13.32 99.957 9.830 9.83 V142R 149.441 12.38 18.50 103.622 12.272 12.72 V142S 170.778 8.73 14.92 117.035 16.900 19.78 V142T 223.936 11.48 25.70 123.650 11.709 14.48 Q143G 143.600 13.88 19.94 98.837 16.096 15.91 Q143K 200.468 14.32 28.70 136.421 20.747 28.30 L144R 136.247 10.71 14.59 111.482 10.182 11.35 L144T 129.746 14.68 19.05 108.923 11.961 13.03 L146P 116.626 1.15 1.34 115.601 3.429 3.96 T147S 142.175 3.93 5.59 130.287 2.605 3.39 T150N 140.724 6.27 8.82 116.923 6.725 7.86 T150S 107.327 6.40 6.87 142.000 6.087 8.64 E151A 103.310 12.11 12.51 126.047 11.783 14.85 E151L 132.125 4.90 6.48 121.830 6.264 7.63 E151S 115.423 6.20 7.15 136.397 4.695 6.40 E151T 128.337 0.00 0.00 110.300 0.000 0.00 E151V 111.531 7.31 8.15 99.647 7.420 7.39 E151W 158.415 1.15 1.83 94.919 0.895 0.85 K152T 149.169 5.57 8.31 136.747 3.558 4.87 K152W 122.313 2.47 3.02 134.039 2.868 3.84 E158S 133.038 0.00 0.00 102.519 0.000 0.00 K162E 67.857 3.51 2.38 41.026 30.000 12.31 L165F 106.283 11.82 12.57 96.667 14.286 13.81 V166Q 155.975 13.35 20.82 117.990 10.953 12.92 V166T 183.384 12.69 23.26 136.882 13.056 17.87 E167D 136.745 10.01 13.69 162.637 3.784 6.15 I169L 140.177 13.19 18.49 122.272 15.528 18.99 K170R 160.710 8.24 13.24 97.128 10.075 9.79 G172A 167.554 7.51 12.59 133.735 7.207 9.64 K173R 106.771 9.80 10.46 134.300 7.489 10.06 L174G 114.130 12.38 14.13 264.368 13.478 35.63 L174N 154.332 13.27 20.48 126.186 18.907 23.86 L174T 124.819 13.06 16.30 144.876 6.098 8.83 N178K 166.871 5.27 8.80 103.154 8.021 8.27 N178R 199.596 4.08 8.15 144.957 3.943 5.72 H193Q 213.585 15.28 32.64 138.113 18.326 25.31 K195T 126.161 22.48 28.36 237.097 15.280 36.23 K195N 130.253 22.38 29.15 96.381 25.487 24.57 K196E 90.574 36.80 33.33 154.091 23.500 36.21 K196R 106.100 13.22 14.02 95.142 17.663 16.81 F204P 83.571 84.62 70.71 82.418 126.000 103.85 N205A 139.223 21.34 29.71 102.031 18.735 19.12 N205E 160.930 19.30 31.06 93.313 18.503 17.27 N205L 107.472 10.56 11.35 0.000 #DIV/0! 8.55 N205T 145.085 10.05 14.58 110.627 13.054 14.44 V206I 189.274 13.17 24.92 111.220 15.575 17.32 K209R 119.794 11.90 14.26 79.535 3.947 3.14 D212N 112.626 2.66 3.00 132.249 5.352 7.08 D212S 122.899 8.35 10.27 147.936 6.841 10.12 D213A 183.830 26.85 49.36 154.770 13.699 21.20 D213M 159.255 6.83 10.88 98.365 6.940 6.83 S215H 109.069 10.04 10.95 78.992 5.758 4.55 S215M 174.883 4.20 7.35

74.943 8.957 6.71 N219I 254.438 8.84 22.49 291.200 11.264 32.80 E220V 131.985 7.43 9.81 113.610 5.909 6.71 T222G 153.033 0.61 0.94 105.454 0.793 0.84 T232F 132.839 12.43 16.51 62.882 19.590 12.32 Q233G 280.488 0.00 0.00 127.368 0.000 0.00 Q234M 95.605 22.31 21.33 80.766 20.283 16.38 S235A 129.818 11.06 14.36 120.916 12.026 14.54 V237C 138.042 0.00 0.00 116.384 0.000 0.00 V237H 122.112 12.43 15.18 145.253 7.407 10.76 V237T 167.105 21.26 35.53 126.020 21.457 27.04 A238E 94.878 8.17 7.76 142.167 6.682 9.50 A238H 59.585 26.09 15.54 204.683 8.345 17.08 T240A 141.283 9.14 12.92 144.667 9.063 13.11 T240Q 162.763 14.76 24.02 120.980 13.776 16.67 R248A 113.237 1.05 1.19 124.650 2.408 3.00 E249V 142.752 15.29 21.83 111.068 16.462 18.28 P257G 125.220 0.78 0.98 112.803 0.677 0.76 K260M 116.690 8.58 10.01 97.396 7.273 7.08 S261A 57.547 67.52 38.86 86.173 54.021 46.55 S261K 161.931 16.05 26.00 116.159 22.820 26.51 S261N 142.901 10.46 14.95 35.529 13.403 4.76 A267T 196.154 35.29 69.23 111.579 38.679 43.16 F273H 122.647 6.00 7.35 119.037 5.973 7.11 F273Y 119.713 7.78 9.32 102.772 9.634 9.90 Q276H 74.908 8.93 6.69 106.393 10.065 10.71 Q276M 98.323 5.64 5.55 104.948 0.000 0.00 Q276R 121.431 10.93 13.27 150.180 8.778 13.18 Q276S 110.643 9.95 11.01 138.696 8.745 12.13 V277A 140.765 6.97 9.82 129.580 5.167 6.70 V277E 175.779 3.75 6.60 195.598 5.222 10.21 V277H 129.434 3.16 4.09 137.684 7.014 9.66 V277K 375.721 13.21 49.63 373.799 12.029 44.96 V277M 137.138 15.05 20.64 112.084 14.851 16.65 V277N 89.645 7.29 6.54 273.386 6.762 18.49 V277Q 119.930 5.70 6.83 116.151 7.772 9.03 V277R 96.071 15.57 14.96 171.465 9.801 16.81 V277S 66.260 7.65 5.07 144.916 4.731 6.86 V277T 101.010 7.99 8.07 143.311 7.788 11.16 L278E 75.408 5.11 3.85 100.179 7.214 7.23 L278G 122.274 6.50 7.94 104.077 7.887 8.21 K279H 138.964 14.99 20.83 123.183 20.090 24.75 V287T 145.345 16.49 23.97 124.738 12.019 14.99 T289S 104.598 0.98 1.02 98.234 0.699 0.69 G291S 184.581 12.17 22.47 119.565 4.156 4.97 G291V 112.807 19.87 22.42 151.039 12.609 19.05 E292C 127.307 8.07 10.27 101.126 8.905 9.01 E292F 137.930 6.17 8.52 132.340 5.840 7.73 E292H 170.153 8.73 14.85 115.501 11.775 13.60 E292R 112.278 12.61 14.16 129.890 11.983 15.56 E292V 163.075 13.28 21.66 133.274 11.847 15.79 T293A 128.197 3.38 4.33 57.524 4.248 2.44 A298G 212.422 8.77 18.63 86.131 9.322 8.03 L307G 117.857 0.00 0.00 91.528 0.000 0.00 S308D 127.652 4.15 5.30 105.846 2.907 3.08 S308K 126.882 1.33 1.69 99.169 0.000 0.00 S308N 170.413 5.67 9.66 139.083 5.907 8.22 I309E 123.847 16.25 20.12 129.940 14.414 18.73 I309G 102.601 7.37 7.56 114.091 6.458 7.37 I309L 153.681 9.58 14.72 106.948 10.905 11.66 I309M 123.425 8.02 9.90 136.797 7.065 9.66 I309N 111.901 6.98 7.81 97.361 6.470 6.30 I309S 169.951 4.11 6.98 551.493 0.862 4.75 I309T 97.936 7.63 7.47 148.864 5.542 8.25 I309V 113.138 1.50 1.70 138.313 3.470 4.80 M310G 167.656 11.44 19.18 110.739 12.916 14.30 M310Q 107.237 27.81 29.82 106.323 28.254 30.04 M313G 138.095 9.77 13.49 109.231 10.141 11.08 M313H 271.914 3.71 10.09 197.024 3.886 7.66 M313K 118.882 0.86 1.02 107.111 0.555 0.59 M313P 103.654 4.98 5.16 132.802 4.516 6.00 M313R 157.272 4.62 7.27 32.845 8.296 2.72 M313T 162.074 7.04 11.40 99.844 7.007 7.00 M313Y 120.038 7.52 9.03 103.011 6.846 7.05 K314S 141.924 9.67 13.73 132.112 9.066 11.98 K314Y 243.011 5.75 13.98 138.931 10.989 15.27 S315A 91.372 16.51 15.08 137.153 9.973 13.68 S315H 151.244 12.06 18.24 105.305 10.867 11.44

S315Y 170.968 30.61 52.33 57.827 39.503 22.84 L317A 123.510 6.97 8.60 132.724 8.395 11.14 L317I 187.477 12.72 23.84 110.696 10.670 11.81 L317K 96.199 3.45 3.31 134.204 3.534 4.74 L317N 127.382 12.02 15.31 121.233 14.528 17.61 L317R 238.501 3.87 9.22 99.467 5.673 5.64 L317S 90.929 15.54 14.13 85.810 6.423 5.51 L317T 145.964 6.96 10.16 154.334 1.087 1.68 L317W 163.704 11.92 19.51 147.606 10.270 15.16 L318D 105.543 17.43 18.40 97.970 16.684 16.35 L318H 99.907 4.29 4.29 124.690 7.363 9.18 L318R 160.469 5.63 9.03 120.872

6.210 7.51 N321R 164.842 9.53 15.71 112.180 8.613 9.66 N321S 102.489 8.29 8.49 108.732 4.534 4.93 E324N 104.618 7.72 8.08 131.265 9.124 11.98 T325E 124.837 14.44 18.02 106.457 10.577 11.26 N328G 197.098 4.15 8.18 109.722 7.233 7.94 N328Y 180.981 10.30 18.64 100.000 10.500 10.50 T335S 107.956 11.57 12.49 125.286 6.288 7.88 Q347A 101.395 10.89 11.04 96.213 11.001 10.58 Q347G 222.459 8.37 18.63 207.054 9.013 18.66 Q349M 99.531 11.98 11.92 108.042 12.330 13.32 Q349R 147.007 11.76 17.29 104.545 13.211 13.81 V351S 130.819 0.00 0.00 100.857 0.000 0.00 I353V 132.334 10.45 13.83 138.025 11.902 16.43 N356H 100.000 8.54 8.54 130.377 3.912 5.10 N356S 51.908 0.00 0.00 125.692 2.516 3.16 S359E 135.589 10.77 14.60 135.104 9.354 12.64 S359H 110.422 0.00 0.00 100.809 0.000 0.00 P367A 167.030 12.94 21.62 127.366 13.153 16.75 P367G 115.683 0.00 0.00 122.642 0.000 0.00 P367K 125.884 5.06 6.36 66.884 10.136 6.78 P367S 74.263 14.39 10.69 88.355 16.433 14.52 D368A 121.623 1.45 1.76 81.646 2.111 1.72 D368E 166.628 9.18 15.30 97.937 11.462 11.23 D368L 108.977 0.00 0.00 109.364 0.969 1.06 D368M 119.744 2.72 3.25 103.662 2.536 2.63 D368R 164.735 10.16 16.74 118.140 11.805 13.95 D368T 107.122 2.87 3.07 126.693 3.366 4.26 N369R 161.693 6.39 10.34 74.366 6.182 4.60 A371F 180.217 6.19 11.16 76.436 5.578 4.26 A371H 957.055 1.81 17.32 89.541 1.697 1.52 A371H 111.143 0.00 0.00 95.589 8.610 8.23 A371K 136.514 12.84 17.53 114.354 12.454 14.24 A371L 695.108 1.51 10.52 107.003 2.215 2.37 A371L 104.327 0.00 0.00 60.232 1.205 0.73 A371R #VALUE! #VALUE! 11.03 #VALUE! #VALUE! 14.06 A371R 121.162 0.00 0.00 97.970 2.587 2.53 A371S 147.672 8.38 12.38 131.555 16.938 22.28 L374P 392.038 5.77 22.63 123.033 7.365 9.06 E375A 88.836 0.00 0.00 134.714 2.050 2.76 E375G 126.880 10.32 13.10 139.030 14.673 20.40 E375R 163.180 13.15 21.45 116.431 19.727 22.97 K376D 113.100 12.36 13.97 165.064 5.049 8.33 K376E 100.000 13.55 13.55 153.016 10.394 15.90 K376Q 125.172 12.75 15.96 90.000 12.057 10.85 K376R 81.687 31.63 25.84 199.112 10.372 20.65 K376T 121.133 14.91 18.06 113.387 5.639 6.39 K376V 124.221 3.19 3.96 118.583 2.547 3.02 K376Y 102.812 9.24 9.50 96.139 12.985 12.48 G377D 110.871 15.72 17.43 132.357 10.550 13.96 G377E 130.445 8.04 10.49 128.402 7.401 9.50 G377H 146.855 8.34 12.25 104.837 10.117 10.61 G377K 185.922 4.42 8.21 119.751 4.989 5.97 G377R 119.708 5.87 7.03 94.749 7.137 6.76 G377S 108.609 6.91 7.51 101.106 7.877 7.96 G377T 112.557 17.14 19.29 109.036 18.279 19.93 F380W 147.077 9.97 14.67 104.881 9.253 9.70 T381S 135.827 13.41 18.21 112.559 10.315 11.61 R383I 527.820 6.33 33.44 98.328 7.522 7.40 R383S 132.894 10.50 13.96 119.466 10.545 12.60 K385A 126.096 4.64 5.85 112.706 0.000 0.00 K385Q 137.629 9.03 12.43 124.892 7.512 9.38 K385V 112.581 5.12 5.76 80.571 2.979 2.40 E389A 306.767 2.13 6.53 224.872 1.824 4.10 E389G 113.253 2.13 2.41 139.901 0.000 0.00 E389L 143.219 14.24 20.40 112.185 12.609 14.15 E389Q 135.807 11.88 16.14 99.738 12.767 12.73 E389S 165.620 0.00 0.00 93.030 0.285 0.27 E392A 112.465 7.27 8.18 155.693 6.376 9.93 E392F 115.619 3.90 4.51 143.781 3.905 5.61 E392Q 112.993 10.53 11.89 93.789 16.705 15.67 E392R 129.528 3.69 4.79 123.407 2.947 3.64 E392V 124.365 7.73 9.61 154.768 6.404 9.91 Q393F 139.966 10.59 14.82 101.647 10.171 10.34 Q393M 139.696 1.60 2.24 86.966 3.086 2.68 S395A 208.246 12.98 27.04 112.714 12.395 13.97 S395H 159.975 12.55 20.07 113.401 10.452 11.85 E396A 131.894 8.42 11.10 128.716 9.777 12.58 E396H 210.364 9.19 19.33 128.571 3.216 4.14 E396Q 122.977 10.06 12.37 95.938 10.263 9.85 E396S 156.267 2.77 4.33 111.753 2.022 2.26 Y399T 130.536 0.00 0.00 122.738 0.050 0.06 Y399V 110.592 15.98 17.68 116.018 17.801 20.65 Y399W 122.500 13.76 16.86 103.346 11.973 12.37 S401A 122.003 13.90 16.96 99.275 13.024 12.93 S401E 125.223 16.30 20.42 128.670 15.000 19.30 S404A 149.379 0.00 0.00 105.443 1.102 1.16 L406F 122.805 0.00 0.00 146.122 0.000 0.00 L406N 152.836 6.36 9.72 131.321 6.705 8.81 S407A 141.351 11.33 16.02 110.376 16.836 18.58 S407D 241.053 11.29

27.22 98.135 10.120 9.93 S407P 143.308 6.85 9.81 121.898 11.088 13.52 A412Q 146.177 9.54 13.94 99.452 8.511 8.46 A412R 140.070 8.92 12.49 123.675 9.390 11.61 A412V 146.804 4.99 7.32 101.739 5.383 5.48 D416L 120.820 17.64 21.31 127.662 15.340 19.58 D418R 117.749 7.59 8.94 112.193 10.721 12.03 A419H 241.224 8.82 21.27 188.179 5.999 11.29 A419K 191.165 10.42 19.91 2022.616 1.523 30.81 D421A 102.111 12.49 12.75 301.584 4.510 13.60 D421H 333.471 10.18 33.95 67.652 86.552 58.55 D421K 124.190 7.62 9.46 102.316 13.562 13.88 D421N 110.806 14.96 16.58 100.116 16.449 16.47 D421Q 104.370 10.72 11.18 143.630 12.400 17.81 D421R 138.783 8.85 12.28 137.964 9.778 13.49 D421S 142.171 11.00 15.64 166.162 8.564 14.23 A425G 74.810 10.61 7.94 120.947 11.137 13.47 G427Q 133.135 2.31 3.08 98.243 8.618 8.47 G427T 125.113 4.81 6.02 119.058 3.956 4.71 V428L 137.044 1.81 2.48 109.390 0.990 1.08 D431E 70.178 26.32 18.47 95.135 20.739 19.73 D431H 186.490 7.32 13.65 95.071 9.941 9.45 D431K 240.835 11.61 27.97 68.277 20.207 13.80 D431L 129.149 10.49 13.54 119.177 11.740 13.99 D431N 138.404 9.79 13.55 125.433 9.246 11.60 D431Q 232.960 10.83 25.23 109.483 10.716 11.73 D431S 78.069 7.52 5.87 88.796 9.135 8.11 F433A 147.286 9.78 14.40 99.486 12.798 12.73 F433H 140.196 13.48 18.90 87.943 16.888 14.85 F433I 108.569 11.30 12.27 86.984 16.616 14.45 F433K 91.159 11.12 10.14 342.290 3.608 12.35 F433R 128.958 10.53 13.58 133.353 9.565 12.75 F433T 161.799 13.66 22.10 134.977 19.229 25.96 F433V 1412.071 1.61 22.69 112.033 17.307 19.39 F433W 149.049 10.46 15.59 113.585 7.530 8.55 P437I 148.880 2.39 3.56 107.028 1.782 1.91 M438A 106.463 10.07 10.72 135.705 10.194 13.83 M438D 105.370 10.16 10.71 113.283 2.590 2.93 M438E 115.061 8.00 9.21 113.782 9.120 10.38 M438L 65.794 10.06 6.62 214.958 6.526 14.03 M438N 130.428 8.06 10.52 100.669 11.889 11.97 M438T 104.058 13.39 13.93 103.691 12.160 12.61 E439A 137.279 11.63 15.97 95.555 14.073 13.45 E439A 154.140 4.72 7.28 147.295 7.415 10.92 E439C 193.243 14.69 28.38 111.719 15.734 17.58 E439K 124.464 13.28 16.52 104.762 10.552 11.05 E439P 118.340 15.59 18.44 87.446 14.998 13.12 E439Q 101.589 10.67 10.84 127.358 10.648 13.56 E439T 110.891 14.36 15.93 122.975 11.322 13.92 T440D 118.877 11.52 13.69 79.518 18.426 14.65 T440H 142.296 4.46 6.34 130.928 7.553 9.89 T440M 84.722 8.83 7.48 86.929 12.774 11.10 T440P 111.931 13.54 15.16 91.205 17.272 15.75 T440S 100.436 11.17 11.22 131.174 9.810 12.87 E441F 129.315 11.25 14.55 110.874 11.410 12.65 E442G 111.216 10.24 11.39 100.210 10.965 10.99 P443E 94.377 5.14 4.85 130.704 6.789 8.87 P443F 146.612 11.22 16.45 97.932 12.322 12.07 P443G 239.171 8.56 20.48 157.960 16.385 25.88 Q444E 81.997 8.54 7.01 160.917 9.561 15.38 Q444H 150.301 8.46 12.71 119.665 10.892 13.03 Q444V 129.822 13.49 17.51 122.591 10.995 13.48 I445M 85.090 17.25 14.68 101.149 15.393 15.57 I445N 106.430 13.89 14.79 87.351 12.945 11.31 I445W 117.213 11.70 13.72 100.037 10.983 10.99 Y447E 99.579 16.55 16.48 108.969 12.849 14.00 Y447G 143.704 13.77 19.79 103.624 11.563 11.98 Y447P 139.152 13.78 19.17 107.737 12.282 13.23 positive 94.998 5.23 4.97 96.871 8.456 8.19 control 105.798 4.48 4.74 108.066 5.246 5.67 (OHO) 100.000 3.33 3.33 82.7780 3.759 4.59 94.762 19.07 18.07 109.539 16.529 18.11 142.024 4.48 6.36 130.947 5.595 7.33 45.115 20.77 9.37 68.017 11.035 7.51 53.324 21.95 11.71 74.253 9.960 7.40 59.581 25.24 15.04 75.872 16.231 12.31 91.844 19.05 17.50 80.371 13.977 11.23 93.828 13.47 12.63 96.630 19.454 18.80 57.773 17.04 9.85 83.536 17.573 14.68 100.000 18.56 18.56 148.226 16.239 24.07 74.325 18.29 13.60 61.119 9.286 5.68 98.132 8.48 8.32 87.677 10.006 8.77 93.817 9.62 9.02 102.223 9.745 9.96 96.922 8.56 8.30 87.993 9.064 7.98 96.648 9.91 9.58 86.891 9.938 8.63 n/a (not available; e.g., beyond detection limit)

~~[0643]~~(548) 2. Summary of Results for F204P

[0644](549) For mutant F204P, the results above of tested supernatant from transient transfection of CHO-SCHO—S cells incubated in the presence of m-cresol in a bHA enzymatic activity assay showed that the F204P mutant protein was highly resistant to 0.4% m-cresol treatment. The results showed that the activity that remained after 4 hours incubation with 0.4% m-cresol at 37.degree.° C. was approximately equal to the activity observed when the enzyme was incubated at either 4.degree.° C. or at 37.degree.° C. in the absence of m-cresol. The positive control (WT PH20-OHO) showed a reduction in activity of 75% and 83% on the day of the assay (as assayed from two different OHO transfections). This demonstrated that the F204P phenophile was able to retain 60% to 90% or greater of its activity above the residual activity of the wildtype PH20 control enzyme.

[0645](550) In order to confirm the stability of F204P upon m-cresol treatment or exposure to increased temperature, a second transfection of F204P was performed in duplicate using CHO-SCHO—S cells, and clarified supernatant was again tested for its stability at 4.degree.° C., at 37.degree.° C. for 4 hours with 0.4% m-cresol and at 37.degree.° C. for 4 hours without 0.4% m-cresol. The results confirmed that the F204P mutant enzyme retained a high amount of hyaluronidase activity after the 4 hour incubation in m-cresol at 37.degree.° C. The results were similar to the results seen in the first screening of the mutant, with F204P retaining anywhere from 57% to greater than 90% of its activity above the residual activity of the wildtype PH20 control enzyme after the 4 hour incubation.

[0646](551) A summary of the enzyme activity of F204P compared to the wildtype control is set forth in Table 13.

(552) TABLE-US-00013 TABLE 13 Summary of Enzyme Activity Remaining Activity Remaining Activity after Activity after 4 h incubation after 4 h incubation (37.degree.° C. + m-cre/ (37.degree.° C. + m-cre/37.degree.° C.) Net % Increase 4.degree.° C.) Net % Increase WT in Activity Over WT in Activity Over Transfection # F204P (OHO) WT (37.degree.° C.) F204P (OHO) WT (4.degree.° C.) 1 73.6% 16.4% 57.2% 86.0% 25.3% 60.7% 2 122.3% 25.2% 97.1% 109.7% 16.6% 93.1%

Example 6

Large Scale Expression and Purification of PH20 Hit Variant

[0647](553) 1. Expression and Purification

[0648](554) HZ24-PH20-IRES-SEAP plasmid DNA containing cDNA encoding one of the variant PH20 was transfected into monolayer CHO-SCHO—S cells as generally described in Example 2. CHO-SCHO—S cells were cultured in shaker flasks using CD-CHO media supplemented with GlutaMAX (8 mM). On the day of transfection, 15 flasks were prepared of approximately 300 mL volume containing the CHO-SCHO—S cells at an approximate density of 1.0.times.×10.sup.6 cells/mL. Each 300 mL flask was transfected using 375 .mu.gµg of plasmid DNA encoding the F204P mutant combined with 375 .mu.LµL of Freestyle MAX transfection reagent. The transfected plasmid DNA had a sequence of nucleotides set forth in SEQ ID NO:4 containing a codon change of TTC to CCT at nucleotide positions 1733-1735, thereby encoding the F204P mutant. The transfected cells were then allowed to remain in culture for 96 hours,

whereupon the cells and media were harvested and pooled. The cells were pelleted by centrifugation (4000.times.×g, 20'), and the supernatant retained for purification of the F204P protein (approximately 4.5 liters).

[0649](555) The crude supernatant was concentrated 10.times.× using a 30 kDa Tangential flow filter (TFF) system (Millipore Pellicon XL, Bimax 30, 200 mL void volume; 50 cm.sup.2 filter surface area) until the volume was approximately 450 mL. The permeate was saved for assay to detect flow through of the F204P protein. A free-flow buffer exchange for the retentate was then performed using 4 liters of buffer (10 mM NaPO.sub.4; 25 mM NaCl, pH 7.2). The volume of the retentate was reduced again to approximately 200 mL, and then the remaining permeate in the system was purged (void volume .about.~200 mL) and the system was flushed using approximately 50 mL of buffer to yield a final concentrated product of approximately 450 mL.

[0650](556) An anti-rHuPH20 affinity column was prepared by coupling antigen affinity purified Rabbit anti-rHuPH20 IgG to CNBr-activated Sepharose 4 Fast Flow (GEHealth catalog No. 17-0981-01). Briefly, 0.7 g of pre-activated Sepharose 4 powder was suspended in 1 mM HCl in a 10 mL glass column for 30 minutes to allow the powder to swell. The solution was drained from the column and washed with 15 gel volumes (about 30 mL) of cold 1 mM HCl by gravity. The column was washed with 5 gel volumes of coupling buffer (0.1M NaHCO.sub.3, 0.5M NaCl at pH 8.3). Next, 5 mg of Rabbit anti-rHuPH20 IgG at >≥1.0 mg/mL in coupling buffer was added to the column at a protein/gel ratio of 2-3 mg/mL gel. The column was rotated head to head at 4.degree.° C. overnight. The flow-through was collected for coupling efficiency determination. The gel was washed with 2 gel volumes of coupling buffer, and then washed and resuspended in 1 M ethanolaminine pH 9.5 for 2 hours at room temperature to block unused activated sites. The gel was washed 6 times with 5 gel volumes per wash alternating coupling buffer and 0.1 NaAc, 0.5M NaCl, pH 4.5. The gel was then washed with 10 gel volumes of TBS (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5). The coupling efficiency was determined (1-post-coupling protein concentration/pre-coupling protein concentration.times.×100%). The antibody coupled gel was stored in TBS with 0.02% NaN.sub.3 at 4.degree.° C.

[0651](557) The concentrated supernatant product was subsequently loaded onto a anti-rHuPH20 affinity column at an approximate rate of 5 mL/min. The elution was performed according to standard procedure using a GE.TM.™ AKTA FPLC purification system (GE Healthcare, Product No. 18-1900-26), whereby the protein was eluted via a low pH glycine wash (0.1 M glycine-HCl, pH 2.5) in 1 mL fractions. Each fraction was immediately neutralized by the addition of 100 .mu.LμL of 1M Tris, pH 7.5.

[0652](558) The eluted protein was assayed by resolving protein bands on a 4-20% SDS-PAGE gradient Tris-glycine gel. SeeBlue.RTM.Plus2SeeBlue® Plus2 Pre-stained MW standards (Life Technologies; Catalog No. LC5925) were used as molecular weight standards, and 50 ng rHuPH20 (as described in Example 1) was used as a positive control. The polyacrylamide gel was stained with Instant Blue to show total protein from each fraction. To confirm the bands on the gel are PH20, the gel was transferred to a PVDF membrane (Invitrogen), which was subjected to Western Blot using a Rabbit anti-PH20 primary antibody generated by immunizing rabbits with rHuPH20 and an HRP-Goat anti-rabbit secondary antibody (Calbiochem, Cat. No. DC03L).

[0653](559) Then, the flow-through from the initial loading of the affinity column was re-loaded onto the column twice due to the low capacity of the affinity column. All fractions containing the protein were then combined resulting in a total volume that was approximately 13 mL. This product was then dialyzed overnight versus four liters of buffer (10 mM NaPO.sub.4, 140 mM NaCl, pH 7.2) using a Slide-A-Lyzer Dialysis Cassette G2 (20,000 MWCO) with a 15 mL capacity. The buffer was then changed and the product dialyzed against a second fresh four liters of the same buffer. The F204P protein was then concentrated using an Amicon Ultra Centrifugation column (Millipore; 10,000 MWCO) to a final volume of approximately 450 .mu.LµL (10 minutes at 4000.times.×g).

[0654](560) 2. Characterization of Protein

[0655](561) The purified protein was characterized for its protein concentration, activity, and purity.

[0656](562) To determine the protein concentration of the purified protein, a quantification ELISA was performed as described in Example 7. Also, hyaluronidase activity was determined as described in Example 3. The protein concentration after centrifugation was estimated to be approximately 400 .mu.gng/mL. The purified protein also was resolved on a 4-20% SDS-PAGE gradient Tris-glycine gel, which was then stained with Instant Blue. The staining results demonstrated that the protein was essentially a single molecular weight protein of approximately 63 kDa, similar to the rHuPH20 control. No appreciable degradative products were detected by this method. Approximate yields of the protein at various timepoints and activity during the purification are described in Table 14.

(563) TABLE-US-00014 TABLE 14 Characterization of Purification Steps Quant Activity Assay Quant ELISA Assay Total Protein Total Specific Purification Volume Activity Activity Conc. Protein Activity Purification Step Volume (mL) (U/mL) (U) (.mu.gµg/mL) (.mu.gµg) (U/.mu.gµg) Supernatant 4500 2.66 11,700 0.046 207 56.5 Conc. after TFF & 450 42 18,900 0.4 178 105.9 TFF & Buffer Exchange Pooled Fractions 0.45 11,741 5283 396 180 35.3 Fractions 5-7 after AC, Dialysis & Conc. - A280

[0657](564) The purity of the purified protein was determined by Reverse Phase HPLC (RP-HPLC). The elution time from the reverse phase column was essentially identical as that observed with the recombinant human hyaluronidase (HUB), and provides a basis for crude estimation of the purity of the sample at approximately 80-90%.

Example 7

Quantification Using ELISA

[0658](565) The quantification of PH20 or variants were performed using an ELISA that captures the protein using a monoclonal anti-rHuPH20 capture antibody. Specifically, one day prior to performing the ELISA, 96-well 4HBX plates were coated with capture antibody (Protein G purified rabbit polyclonal anti-PH20 antibody generated by immunizing rabbits with rHuPH20; 1 mg/mL stock) at 1 .mu.gng/mL in 100 mM phosphate (pH 7.2) in a total volume of 100 .mu.LµL per well. The plates were stored at 4.degree.° C. overnight. On the next day, the plates were washed 5.times.× with 1.times.×PBS at 300 .mu.LµL/well with a plate washer. After

each wash, the plated were patted dry on paper towels. Then, the plates were blocked with 200 .mu.LµL PBS containing Tween 20 (1.times.×PBST) per well at room temperature for 1 hour.

[0659](566) The standards and samples were added to the plate. For generation of the standard, a 1 mg/mL stock of rHuPH20 (Example 1) was freshly diluted to 50 .mu.gng/mL in HEPES pH 7.4 assay buffer as an intermediate stock. Then, for the standards, the 50 .mu.gng/mL stock was diluted in duplicates into 360 .mu.LµL of 0.5.times.×PBST at 300 ng/mL for the first standard (first row). For the other standard rows, 240 .mu.LµL 0.5.times.×PBST were added to each well, and 1:3 serial dilutions made. For the transfected supernatant samples, 360 .mu.LµL per well was added in duplicate into the first row, and each were also serially diluted as described above into 0.5.times.×PBST. For purified samples, 100 .mu.LµL was added per well. The plates were incubated for 2 hours at room temperature. After incubation, the plates were washed 5.times.× with 1.times.×PBST at 300 .mu.LµL/well using a plate washer. After each wash, the plates were patted dry on paper towels.

[0660](567) An HRP-conjugated anti-PH20 antibody was prepared for detection using an HRP conjugation kit (Pierce, Thermo-Fisher; Catalog No. 31489). 1 mg of a Protein G purified rabbit polyclonal antibody generated by immunizing rabbits with rHuPH20 was diluted in 1 mL PBS and 1 mL of 2.times.× carbonate kit buffer. Next, 100 .mu.LµL of peroxidase were added to 1 mL of the above antibody solution and incubated at room temperature for 1 hour. Then, 10 .mu.LµL NaBH.sub.4 stock was added in a fume hood, and the sample incubated at room temperature for 20 minutes. To quench the reaction, 20 .mu.LµL of ethanolamine was added and incubated at room temperature for 15 minutes. To this, 1/25 volume 5% human serum albumin (0.1 mL syringe) was added to give a 2 mg/mL albumin stock reaction. The pH was adjusted to about 7.9 by addition of 250 .mu.LµL of 1 M Tris pH 7.4. The concentration of the stock was 400 .mu.gµg/mL. The stock solution was further diluted 1/10 in PBS Tween20 (0.05%) containing 0.5% human serum albumin and preservatives, and then was sterile filtered. The stock was stored at 4.degree.° C. or was frozen at -20.degree.−20° C.

[0661](568) Antibodies were detecting using the HRP-conjugated anti-PH20 antibody that was diluted 1000.times.× into 0.5.times.×PBST. 100 .mu.LµL of the diluted antibody was added to all wells of the plate and the plate incubated for a further 2 hours at room temperature. After incubation, the plates were washed 5.times.× with 1.times.×PBST at 300 .mu.LµL/well using a plate washer. After each wash, the plates were patted dry on paper towels. Then, 100 .mu.LµL of TMB substrate were added to each well and the reaction was stopped after 5-10 minutes by adding 100 .mu.LµL of stop solution per well. The plate was read at OD.sub.450.

Example 8

Determination of Enzymatic Activity of PH20

[0662](569) Enzymatic activity of PH20 in samples such as cell cultures, purification fractions and purified solutions was determined using a turbidimetric assay, which is based on the formation of an insoluble precipitate when hyaluronic acid binds with cetylpyridinium chloride (CPC). The activity is measured by incubating PH20 with hyaluronan for a set period of time (30 minutes) and then precipitating the undigested hyaluronan with the addition of CDC. The turbidity of the resulting sample is measured at 640 nm. The decrease in turbidity resulting from

enzyme activity on the hyaluronan substrate is a measure of the PH20 enzymatic activity. The method is run using a calibration curve generated with dilutions of a PH20 assay working reference standard (rHuPH20 standard generated as described in Example 1), and sample activity measurements are made relative to this calibration curve.

[0663](570) Dilutions of the sample and standards were prepared in Enzyme Diluent Solution (70 mM NaCl, 0.1% human serum albumin [HSA], 0.67 g/L gelatin hydrolysate in 25 mM PIPES buffer, pH 5.5). The samples were diluted to an appropriate concentration. Hyaluronic acid (HA, average MW of 20-50 kDa) from Lifecore Biomedical (Chaska, Minn.) also was prepared at 1 mg/mL in substrate solution that contains 25 mM PIPES, 70 mM NaCl at pH 5.5. Equal amounts of the above two solutions were mixed to prepare a 1 mL reaction mixture and incubated at 37.degree.° C. for 30 min. The reaction was stopped by addition of 4 mL of Cetylpyridinium Chloride Solution (CPC, 5.0 mg/mL). After brief vortexing, the turbidity of the sample mixture was read at 640 nm and the activity was determined by fitting against a standard curve. Specific activity (Units/mg) was calculated by dividing the enzyme activity (U/mL) by the protein concentration (mg/mL).

Example 9

Stability of F204P-PH20 Variant in Preservative

[0664](571) To confirm the screening results, an amount estimated to be about 450 U/mL of the purified F204P protein as described in Example 6 was formulated in 10 mM sodium phosphate, pH 6.5, 120 mM NaCl, 10 mM methionine, 0.01% Pluronic F-68, 0.1% phenol and 0.15% m-cresol. A test article that also contained an amount estimated to be about 450 U/mL wild type rHuPH20 (generated as described in Example 1) in the same formulation was also prepared to serve as a control. Each formulation solution was aliquottedaliquoted in 0.5 mL and filled into 2 mL USP Type I borosilicate glass with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 5.degree.° C., 30.degree.° C. or 37.degree.° C. Samples were withdrawn from the incubator at various times and enzymatic activity was measured as described in Example 8.

[0665](572) The results of the enzymatic activity measurements are shown in Table 15. As can be seen, the rHuPH20 wild type control showed a rapid decrease in activity when incubated at 37.degree.° C. in the presence of phenolic preservatives. In contrast, the F204P mutant showed no significant loss in activity throughout the study. The results also show that activity of PH20 is retained after incubation for up to 4 weeks at 5.degree.° C. and 30.degree.° C. compared to the activity of the rHuPH20 wildtype control not containing the mutation. These results confirm that F204P tolerates EPB level of preservative (0.1% phenol and 0.15% m-cresol) and is stable at 37.degree.° C. for at least up to 6 days at at 5.degree.° C. and 30.degree.° C. for greater than one month.

(573) TABLE-US-00015 TABLE 15 Stability of rHuPH20 wildtype and F204P mutant incubated at with preservative PH20 relative PH20 relative PH20 relative activity activity activity (%) at 5.degree. C.activity (%) at 30.degree.PH20 relative activity 5° C. 30° C. (%) at 37.degree.° C. ID T0 2 w 4 w 6 d 2 w 4 w 2 d 4 d 6 d F204P 100 ---- 91.8 84.1 100 96.6 105 91.1 95.9 wildtype control 100 ---- 81.9 66.7 61.7 60.5 48.6 29.6 15.2

Example 10

Stability of F204P-PH20 Variant in Insulin Coformulation

[0666](574) The PH20 variant F204P was tested for its stability in a coformulation containing an insulin analog (insulin aspart or insulin lispro).

[0667](575) In the tested coformulations, the insulin lispro was a commercial product (Insulin Lispro: Eli Lilly Humalog.RTM.® (insulin Lispro) 100 U/mL, Lot A572364).

[0668](576) In the tested coformulations, the insulin aspart analog was a reprocessed aspart prepared by pooling 12 vials (10 mL each) of a commercial product (Insulin Aspart: Novo Nordisk, NovoRapid.RTM.® (insulin Aspart), Lot XS60195), which was then concentrated using an Amicon Ultracel-10 K column concentrator until the final concentration was about 5 times the original concentration. The insulin analog was precipitated by addition of 1 M sodium acetate, pH 5.3 and 30 mM zinc chloride (ZnCl.sub.2, EMD, Cat No. ZX0065-1) at 1/10 of the protein solution volume. The solution was placed on ice for 30 minutes followed by centrifugation at 5600 rpm for 20 minutes in an Avanti J-E Centrifuge with JS-5.3 swinging bucket rotor (Beckman Coulter). The supernatant was decanted and the pellet was resuspended and washed with 20 mM sodium acetate, 2 mM zinc chloride, pH 5.5 solution. The resuspended solution was centrifuged as described above. The washing step was repeated a total of 5 times. A final wash was performed with 20 mM sodium acetate, pH 5.5 to remove all traces of zinc chloride. The resulting protein paste was dissolved with water containing 20 mM HCl. After complete dissolution, 250 mM Tris, pH 10.7 was added to a final Tris concentration of 20 mM. The pH of the resulting solution was adjusted such that the insulin analog was formulated as described below and the protein concentration was adjusted to about 15-20 mg/mL. An insulin analog prepared in this way typically had a yield of about 90%, with a residual preservative concentration at less than 100 times the starting material.

[0669](577) Briefly, three (3) formulations were generated each containing 600 Units (U) of PH20-F204P or wildtype rHuPH20 (generated as described in Example 1) for a total of 6 formulations as set forth in Table 16:

(578) TABLE-US-00016 TABLE 16 Summary of Insulin Formulations Buffer Tonicity PreservativesSur- API Tris/ modifier Anti-Ox Metal Surfactant m- factant Preservatives PH20 Analog ID pH NaPO.sub.4 HCl NaCl Methionine Glycerin Zn F68 Phenol Cresolm-Cresol (U/mL) (mg/mL) F1. Humalog + 7.0-7.8 13.2 mM 173.7 mM 0.242 mM 0.315% 600 3.5 Humalog + F204P F2. Humalog + 7.0-7.8 13.2 mM 173.7 mM 0.242 mM 0.315% 600 3.5 Humalog + wt F3. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 F204P F4. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 wt F5. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 F204P F6. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 wt

[0670](579) Each formulation solution was dispensed in 0.5 mL aliquots into 2 mL USP Type I borosilicate glass vials with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 5.degree.° C., 30.degree.° C. and 37.degree.° C. Samples were withdrawn from the

incubator at scheduled time points for enzymatic activity measurements as described in Example 8.

[0671](580) The results of the enzymatic activity measurements for samples incubated at 37.degree.° C., 30.degree.° C. and 5.degree.° C. are shown in Tables 17-19, respectively. At 37.degree.° C., the enzymatic activity of samples containing wildtype rHuPH20 (F2, F4 and F6) were almost totally lost within two days of incubation. In contrast, after 6 days incubation at 37.degree.° C., formulation F3 and F5, which contains PH20-F204P, lost only about 10% and 30%, respectively. The PH20-F204P formulated in commercial Humalog (F1) lost most of its activity within 2 days at 37.degree.° C. most likely due to the lack of NaCl in the formulation.

[0672](581) A similar trend for enzymatic activities of ampoules incubated at 30.degree.° C. was noted between the PH20-F204P and rHuPH20. For formulations that contain an EPA preservative level, the differences between wild type and F204P were dramatic (Table 17; F1 and F5 vs. F2 and F6). When the preservative concentration was reduced to an EPB level (F3 and F4),

[0673] the F204P still outperformed wildtype rHuPH20, although there was slightly higher rHuPH20 stability compared to EPA conditions. In both EPA and EPB preservative levels, PH20-F204P was able to maintain its activity up to 14 days at 30.degree.° C. when 100 mM of NaCl was included in the formulation.

(582) TABLE-US-00017 TABLE 17 Enzymatic activity of rHuPH20 wild type and F204P mutant incubated at 37.degree.° C. PH20 activity U/mL, (% of remaining activity) Initial ID Activity 2 d 4 d 6 d 2 w F1. Humalog + F204P 583 (100%) 61 (10%) 15 (3%) 10 (2%) -- F204P F2. Humalog + wt 439 (100%) 4 (1%) F3. Aspart + F204P 625 (100%) 613 (98%) 496 (79%) 570 (91%) 532 (85%) F204P F4. Aspart + wt 566 (100%) 58 (10%) 24 (4%) 4 (1%) F5. Aspart + F204P 657 (100%) 484 (74%) 462 (70%) 478 (73%) 360 (55%) F204P F6. Aspart + wt 596 (100%) -1 −1 (0%)

(583) TABLE-US-00018 TABLE 18 Enzymatic activity of rHuPH20 wild type and F204P mutant incubated at 30.degree.° C. PH20 activity U/mL, (% of remaining activity) Initial ID Initial Activity 6 d 2 w 4 w F1. Humalog + F204P 583 (100%) 345 (59%) 250 (43%) 111 (19%) F204P F2. Humalog + wt 439 (100%) 1 (0%) 16 (4%) -1 wt −1 F3. Aspart + F204P 625 (100%) 601 (96%) 650 (104%) 579 (93%) F204P F4. Aspart + wt 566 (100%) 428 (76%) 390 (69%) 277 (49%) wt F5. Aspart + F204P 657 (100%) 632 (96%) 655 (100%) 561 (85%) F204P F6. Aspart + wt 596 (100%) 145 (24%) 65 (11%) 9 (1.5%) wt

(584) TABLE-US-00019 TABLE 19 Enzymatic Activity at 5.degree.° C. PH20 activity (U/mL) at 5.degree.° C. ID Initial Activity 2 w 4 w F1. Humalog + F204P 583 544 565 F2. Humalog + wt 439 428 404 F3. Aspart + F204P 625 647 607 F4. Aspart + wt 566 580 496 F5. Aspart + F204P 657 695 574 F6. Aspart + wt 596 583 519

Example 11

[0674] Stability of V58R-PH20 in Insulin Coformulation

[0675](585) A. Stability of V58R-PH20

[0676](586) The PH20 variant V58R was expressed in CHO-SCHO—S cells as described in Example 2 or Example 6. The transfected plasmid DNA had a sequence of nucleotides set forth in SEQ ID NO:4 containing a codon change of GTG to CGG at nucleotide positions 1295-1297, thereby encoding the V58R mutant. The V58R mutant was tested for its stability in a coformulation containing insulin aspart (insulin aspart analog prepared as described in Example 10) and under EPA or EPB preservative levels. Briefly, four (4) formulations were generated each containing 600 Units (U) of PH20-V58R or wildtype rHuPH20 (generated as described in Example 1) as set forth in Table 20. Formulations F1 and F2 represent the EPB preservative levels while formulations F3 and F4 represent the EPA preservative levels.

(587) TABLE-US-00020 TABLE 20 Summary of Insulin Formulations Buffer Tonicity Sur- API Tris/Buffer modifier Anti-Ox Metal Surfactantfactant Preservatives PH20 Analog ID pH NaPO.sub.4 Tris/HCl NaCl Methionine Glycerin Zn F68 Phenol m-Cresol (U/mL) (mg/mL) F1. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 V58R F2. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 rHuPH20 wt F3. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 V58R F4. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 rHuPH20 wt

[0677](588) Each formulation solution was dispensed in 0.5 mL aliquots into 2 mL USP Type I borosilicate glass vials with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 30.degree.° C. and 37.degree.° C. Samples were withdrawn from the incubator at scheduled time points for enzymatic activity measurements as described in Example 8.

[0678](589) The results of the enzymatic activity measurements for samples incubated at 37.degree.° C. and 30.degree.° C. are shown in Table 21 and Table 22. At 37.degree.° C., the enzymatic activity of samples containing wildtype rHuPH20 (F2 and F4) were almost totally lost within two days of incubation. In contrast, after 6 days incubation at 37.degree.° C., formulations F1 (EPB) and F3 (EPA), containing V58R-PH20, lost only about 25% and 40% activity, respectively. At 30.degree.° C., the enzymatic activity of samples containing wildtype rHuPH20 also was dramatically reduced in the presence of EPA or EPB preservatives levels within one month of incubation, although there was a slightly less dramatic loss in activity in the presence of EPB preservative levels. In contrast, for V58R-PH20, there was no loss of enzymatic activity for either tested formulation up to 1 month.

(590) TABLE-US-00021 TABLE 21 Enzymatic activity of rHuPH20 wild type and V58R mutant incubated at 37.degree.° C. PH20 activity U/mL Formulation Initial Activity 2 d 4 d 6 d F1. Aspart + V58R 1350 1099 1094 1006 F2. Aspart + rHuPH20 wt 677 53 -3 - −3 — F3. Aspart + V58R 1189 793 581 464 F4. Aspart + rHuPH20 wt 744 12 -9 - −9 —

(591) TABLE-US-00022 TABLE 22 Enzymatic activity of rHuPH20 wild type and V58R mutant incubated at 30.degree.° C. PH20 activity U/mL Formulation Initial Activity 2 weeks 4 weeks F1. Aspart + V58R 1350 1368 1208 F2. Aspart + rHuPH20 wt 677 422 256 F3. Aspart + V58R 1189 1228 1171 F4. Aspart + rHuPH20 wt 744 21 -5

−5 [0679] B. Comparison of Stability of F204P and V58R

[0680](592) The PH20 variant V58R-PH20 was compared to F204P for its stability in a coformulation containing insulin aspart (insulin aspart analog prepared as described in Example 10) and under EPA or EPB preservative levels. Briefly, eight (8) formulations were generated as set forth in Table 23. Formulations F1-F4 represent the EPB preservative levels while formulations F5-F8 represent the EPA preservative levels. Formulations F3 and F4 and formulations F7 and F8 were identical and represent the wildtype control formulations formulations used for the EPB or EPA studies, respectively.

(593) TABLE-US-00023 TABLE 23 Summary of Insulin Formulations Buffer Tonicity Anti-Preservatives API Tris/ modifier Anti-OxOx Metal Surfactant m- PH20 Analog ID pH NaPO.sub.4 HCl NaCl Methionine Glycerin Zn F68 Phenol Cresol U/mL mg/mL F1. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 V58R F2 Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 F204P F3. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 rHuPH20 wt(1) F4. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.100% 0.150% 600 3.5 rHuPH20 wt(2) F5. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 V58R F6 Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 F204P F7. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 rHuPH20 wt(1) F8. Aspart + 7.3 30 mM 100 mM 5 mM 0.010% 0.315% 600 3.5 rHuPH20 wt(2)

[0681](594) Each formulation solution was dispensed in 0.5 mL aliquots into 2 mL USP Type I borosilicate glass vials with a chlorobutyl rubber stopper and an aluminum seal. The vials were incubated at 30.degree.° C. and 37.degree.° C. Samples were withdrawn from the incubator at scheduled time points for enzymatic activity measures as described in Example 8.

[0682](595) The results show that the percentage hyaluronidase activity in the tested formulations after preincubation at 37.degree.° C. was slightly greater for both PH20 mutants when formulated in EPB and not EPA preservative levels. While the percent of activity remaining was greater than 80% for both tested mutants after 6 days incubation in formulations containing EPB preservative levels, it was less in the presence of EPA preservative levels. For example, the activity remaining at 6 days in EPA preservative levels was slightly less than 80% after 6 days for F204P-PH20, while it was only about 40% for V58R-PH20. Hence, the results also show that at 37.degree.° C., V58R-PH20 is somewhat less stable than the F204P-PH20, in particular in a formulation with EPA preservative levels. After incubation at 30.degree.° C. for at least a week, the F204P-PH20 and V58R-PH20 were stable and exhibited almost 100% initial activity in the presence of both EPA and EPB preservative levels. In contrast, rHuPH20 exhibited only about 40% of its initial activity after 4 weeks at 30.degree.° C. in the presence of EPB preservative levels, while it exhibited no detectable activity after 4 weeks at 30.degree.° C. in the presence of EPA preservative levels.

Example 12

Expression of F204P-PH20 Using a Lentivirus Expression Vector

[0683](596) A lentivirus expression vector, pLV-EF1a-PH20(F204P)-IRES-GFP-Bsd was generated containing a codon-optimized mutant hyaluronidase cDNA encoding F204P-PH20. The sequence of pLV-EF1a-PH20(F204P)-IRES-GFP-Bsd is set forth in SEQ ID NO:925. The pLV-EF1a-PH20(F204P)--IRES-GFP-Bsd vector contains an ampicillin resistance gene (AmpR)

located at nucleotides 8611-9471, an EF1a promoter at residues 1933 to 2327, an IRES at residues 4786-5370, a GFP-Bsd at residues 5394-6527 and nucleotides encoding F204P-PH20 at residues 3369-4781.

[0684](597) Lentivirus was produced as described in Bandaranayake et al. ((2011) Nucleic Acids Research, 39:e143). Briefly, 293T cells (ATCC) were plated at 6.times.×10.sup.6 cells onto 10 cm tissue culture plates. After 24 hours, 6 .mu.gµg of psPAX2 (SEQ ID NO:926; Addgene plasmid No. 12260), 3 .mu.gµg of PMD2.G (SEQ ID NO:927; Addgene plasmid #12259) and 9 .mu.gµg lentiviral vector plasmid pLV-EF1a-PH20(F204P)-IRES-GFP-Bsd were mixed in 1.5 mL Opti-MEM (Life Technologies). 45 .mu.LµL of Lipofectamine 2000 (LF2000; Life Technologies) were diluted into 1.5 mL Opti-MEM (Life Technologies). The DNA and LF2000 were mixed gently, and incubated at room temperature for 20 minutes to allow the DNA and lipid to form complexes. In the meantime, the overnight culture medium was replaced with 5.0 mL DMEM+10% FBS without antibiotics. A volume of 3.0 mL containing the DNA-LF2000 complexes were added to the 293T cells. The medium containing the DNA-LF2000 complexes was replaced with 10 mL complete medium at 12-16 hours post-transfection. The supernatant was collected at 48 hours post-transfection and the medium was transferred to a polypropylene storage tube. The virus-containing medium was spun at 1300 rpm for 5 minutes to pellet any 293T cells that were carried over during collection. The supernatant was carefully transferred to a sterile polypropylene storage tube.

[0685] CHO-S(598) CHO—S cells (Invitrogen) were grown in CHO-SCHO—S media (Invitrogen) with shaking at 120 rpm at 37.degree.° C. and 5% CO.sub.2 in vented 125-mL shake flasks (Nalgene). For transduction, CHO-SCHO—S cells were added to wells of a six-well plate at 2*×10.sup.6 cells per well in 2 ml of CHO-SCHO—S media containing 4 .mu.gng/mL hexadimethrine bromide at a final concentration of 4 .mu.gµg/mL (Polybrene; SIGMA). Virus was added to each well at a multiplicity of infection (MOI) of 10 and the cells were incubated with shaking (120 rpm) at 37.degree.° C. and 5% CO.sub.2 for 6 hours. The cells were then harvested and pelleted by low speed centrifugation (500.times.×g, 5 min).- The transduction medium was removed and replaced with 10 mL of fresh CHO-SCHO—S medium (Invitrogen) supplemented with GlutaMax (50 mL/liter) and transferred to a T-25 flask. Three days post infection, blasticidin (Invitrogen) was added to the growth medium at a concentration of 1 .mu.gµg/mL. The medium was changed regularly at 3-4 day intervals, and the cells were transferred to a T75 flask for expansion. Two weeks after the initial infection, the cells were expanded to shaker flasks and maintained in culture using medium containing 1 .mu.gng/mL blasticidin. F204P-PH20 protein secreted into the CHO-SCHO—S medium was collected and purified by affinity chromatography using an anti-rHuPH20 affinity column as described in Example 6. The protein was prepared in standard API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5).

Example 13

Analysis of Secondary Structure and Melting Temperature

[0686](599) The secondary structure and melting temperature of the PH20 variant F204P was tested and compared to wild-type rHuPH20 (generated as described in Example 1) to further assess stability of the variant. The secondary structure was tested by circular dichroism. A Jasco

J-~~810-150S~~810-1505 equipped with PTC-~~424S~~-4245 was employed for the CD spectral measurement and the CD spectra were collected by Spectra Manager (Version 1.5, Jasco). Procedures for instrumental set up and data collection are described in Table 24.

(600) TABLE-US-00024 TABLE 24 CD Spectroscopy Operation Conditions Parameters Conditions Nitrogen flow rate 25 ft.sup.3/h Sample temperature 30-75~~.degree.~~° C. Sample concentration Approx. 0.1 mg/mL Cell pathlength 1 mm Wavelength 220 nm Data pitch 1~~.degree.~~° C. Delay time 60 seconds Temperature slope 1~~.degree.~~° C./min Sensitivity standard Response 4 seconds Band width 1 nm

~~[0687]~~(601) 1. Sample Preparation and Measurement

~~[0688]~~(602) Two hundred (200) ~~.mu.L~~µL of a 0.1 ~~mgmL~~mg.Math.mL protein sample diluted in McIlvaine's buffer (McIlvaine (1921) JBC 49:183) adjusted to pH 6.5 were prepared. A series of samples of the F204P variant were also generated that varied in pH by adjustment using McIlvaine's buffer to a pH range from 5.0 to 7.5 as set forth in Table 25. In addition, samples also were generated by adjusting the NaCl concentration to 17.5 mM to 140 mM as set forth in Table 26. Samples were filtered using a 0.2 ~~.mu.m~~µm syringe filter prior to measurement. Similar samples were generated for rHuPH20. Then, 200 ~~.mu.L~~µL samples were transferred to a rectangular cuvetted having a 1 mm width and seated on Jasco J-810 spectropolarimeter. CD spectra of the samples were collected under the conditions described in Table 20. The melting temperature (T.sub.m) was calculated using Spectra Manager (v 1.5, Jasco) from the CD spectral intensity measured at the temperature range from 30~~.degree.~~° C. to 75~~.degree.~~° C. The cuvettes were cleaned by Chromerge~~.RTM.~~® cleaner (C577-12, Fisher scientific) between individual sample loading and after the run.

(603) TABLE-US-00025 TABLE 25 Sample pH and concentration F204P concentration Target pH Actual pH F204P (~~.mu.L~~µL) Buffer (~~.mu.L~~µL) (mg/mL) 5.0 4.92 25 175 0.1 5.5 5.38 25 175 0.1 6.0 5.99 25 175 0.1 6.5 6.49 25 175 0.1 7.0 7.00 25 175 0.1 7.5 7.5 25 175 0.1

(604) TABLE-US-00026 TABLE 26 Sodium Concentration in Samples at pH 6.5 Target NaCl F204P concentration NaCl, 2.8M F204P Buffer at pH concentration (mM) (~~.mu.L~~µL) (~~.mu.L~~µL) 6.5 (~~.mu.L)~~µL) (mg/mL) 17.5 0.00 25 175 0.1 50.0 2.32 25 172.7 0.1 75.0 4.11 25 170.9 0.1 100.0 5.89 25 169.1 0.1 140.0 8.75 25 166.3 0.1

~~[0689]~~(605) 2. Results

~~[0690]~~(606) The results show that the secondary structure of F204P is similar to rHuPH20. As a function of temperature, circular dichroism showed that a change in the absorption was measured with increasing temperatures. As a function of pH, the T.sub.m distribution was closely comparable for both F204P and rHuPH20 and the highest T.sub.m for each was obtained between pH 5.5 and pH 6.0. The results, however, showed that T.sub.m of the F204P variant was approximately 9~~.degree.~~° C. higher at all tested ranges than wildtype rHuPH20. This result indicated that the F204P mutant is more stable against thermal stress conditions. As a function of salt, the results show that the F204P and wildtype rHuPH20 both exhibited an increasing T.sub.m with higher salt concentration, showing that both have a proportional inclination toward salt concentration.

Example 14

Assessment of Enzymatic Activity in an Intradermal ~~Tyrpan~~Trypan Blue Dispersion Assay

~~[0691]~~(607) Spreading activity of the PH20 variant F204P was assessed using a dye dispersion in vivo assay. Briefly, purified PH20 variant F204P (prepared as described in Example 12) and wild-type rHuPH20 (prepared as described in Example 1) were both formulated in API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5) at a concentration of 10,000 U/mL. The stocks were further diluted to three target concentrations of 1000, 100 and 10 U/mL by serial 1:10 dilutions in API buffer. Purified proteins (either rHuPH20 or F204P-PH20) were diluted 1:1 with 0.4% Trypan Blue (0.4% liquid solution; Catalog No. 15250, Invitrogen) to give a final concentration of 5, 50 and 500 U/mL protein, each containing 0.2% trypan blue. A vehicle control (API buffer) also was prepared. Forty-two (42) female NCr nu/nu homozygous mice were used in the study with six mice used per group as set forth in Table 27.

(608) TABLE-US-00027 TABLE 27 Summary of Treatment Groups for Dye Dispersion Study Final Dose with Trypan Injection No. of Test Blue Trypan ~~Injection~~Volume Group Mice ~~Test~~ Article (Units/mL) Blue ~~Volume~~ (mL) 1 6 Control 0 0.2% 0.04 2 6 rHuPH20 5 0.2% 0.04 3 6 rHuPH20 50 0.2% 0.04 4 6 rHuPH20 500 0.2% 0.04 5 6 F204P-PH20 5 0.2% 0.04 6 6 F204P-PH20 50 0.2% 0.04 7 6 F204P-PH20 500 0.2% 0.04

~~[0692]~~(609) Forty (40) ~~.mu.L~~µL of samples were administered by a single intradermal injection. The area of dye dispersion was measured at 2.5, 5, 10, 15 and 20 minutes post-injection and was recorded by photographic imaging by photograph of the injection site with a Nikon D90 digital camera with 60 mm prime micro-lens. A laser distance meter (Leica D3) was used to accurately position the camera at a pre-determined distance from the Trypan Blue dye area on the animal. The area of the dye was determined using Image-Pro Analyzer 7.0 (MediaCybernetics, Inc). The calculated areas were expressed as mm.sup.2.

~~[0693]~~(610) The results are set forth in Table 28. The results showed that the dispersion activity of the PH20 variant F204P was substantially identical to the dispersion activity of rHuPH20. The ability to increase the area of dye dispersion was dose-dependent, with both proteins having greatest activity at 500 U/mL. The results also showed that the area of dye dispersion increased with time post-intradermal injection. The areas of dye dispersion of rHuPH20 and F204P-PH20 were significantly greater than the areas of dye dispersion for the controls (p~~<~~≤0.05) at all time points when formulated at all concentrations (5, 50 and 500 U/mL) with the exception of rHuPH20 at the lowest concentration (5 U/mL). When compared to each other, rHuPH20 and F204P-PH20 showed similar dispersion effects, although there was a significant difference in dispersion between the two groups at 5 U/mL and 500 U/mL but not at 50 U/mL. In sum, the results show that both rHuPH20 and F204P-PH20 provided a statistically significant increase in the area of dye dispersion compared to the vehicle control.

(611) TABLE-US-00028 TABLE 28 Trypan Blue Dispersion Group Area (mm2) Avg. (n = 6) 2.5 min 5 min 10 min 15 min 20 min 1: Control 37.44 ~~.+-.~~± 2.81 38.16 ~~.+-.~~± 3.33 43.71 ~~.+-.~~± 2.12 45.70 ~~.+-.~~± 2.38 48.77 ~~.+-.~~± 2.14 2: rHuPH20 36.68 ~~.+-.~~± 2.83 42.31 ~~.+-.~~± 2.57 45.41 ~~.+-.~~± 2.75 46.72 ~~.+-.~~± 3.35 49.61 ~~.+-.~~± 2.97 (5 U/mL) 3: rHuPH20 39.24 ~~.+-.~~± 1.20 44.90 ~~.+-.~~± 1.44 46.96 ~~.+-.~~± 1.70 50.08 ~~.+-.~~± 2.07 53.50 ~~.+-.~~± 1.59 (50 U/mL) 4: rHuPH20 44.72 ~~.+-.~~± 1.35 50.21

.+-.± 1.92 57.47 .+-.± 1.29 59.77 .+-.± 1.25 57.17 .+-.± 3.28 (500 U/mL) 5: F204P 39.65 .+-.± 1.53 46.09 .+-.± 2.73 48.07 .+-.± 1.43 52.54 .+-.± 2.01 54.11 .+-.± 1.01 (5 U/mL) 6: F204P 38.10 .+-.± 1.92 47.07 .+-.± 2.12 51.48 .+-.± 2.14 55.24 .+-.± 1.90 58.34 .+-.± 2.89 (50 U/mL) 7: F204P 46.58 .+-.± 1.67 54.06 .+-.± 2.52 58.96 .+-.± 1.85 64.37 .+-.± 1.72 64.44 .+-.± 2.17 (500 U/mL)

Example 15

Assessment of Enzymatic Activity by Dermal Barrier Reconstitution

[0694](612) Activity of F204P-PH20 was assessed and compared to rHuPH20 to measure the amount of time required for the dermal barrier to reconstitute itself after intradermal hyaluronidase administration. Dermal reconstitution was evaluated by comparing the duration of the hyaluronidase spreading activity as assessed by monitoring the area of diffusion of 0.4% Trypan Blue over time. The proteins used in the study were purified PH20 variant F204P (prepared as described in Example 12) and wild-type rHuPH20 (prepared as described in Example 1) that were both formulated in API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5). Vehicle (API buffer) was used as a control. Male NCr nu/nu homozygous mice were used in the study with three animals per time point for a total of fifteen mice used per group as set forth in Table 29.

(613) TABLE-US-00029 TABLE 29 Summary of Treatment Groups for Dermal Barrier Reconstitution Study Injection No. of Time Points Test Final Dose Volume Group Mice (h) Test Article (Units/mL) (mL) 1 15 0.5, 1, 4, 24, Control 0 0.04 48 2 15 0.5, 1, 4, 24, rHuPH20 100 0.04 48 3 15 0.5, 1, 4, 24, F204P 100 0.04 48

[0695](614) All mice received two intradermal doses of vehicle control or rHuPH20 or F204P-PH20 at 100 U/mL in 0.04 mL at study time 0. The same control or test article was injected on the opposing lateral sides of each animal (right, R; left, L). Injection sites were marked with a permanent marker. Trypan Blue Stain (0.4% liquid solution; 15250, Invitrogen) was administered at a volume of 0.04 mL by intradermal injection at the same injection site at 0.5, 1, 4, 24 and 48 hours post-injection of test article or control. At 5 and 20 minutes post-injection of the Trypan Blue Stain, the area of the dye at the injection site was measured by digital imaging of the region as described in Example 14.

[0696](615) The results are set forth in Table 30. The results show that when the area of dye dispersion was measured at various time points after administration of the test article or control, there was a statistically significant increase in the area of dye dispersion at 30 min and 1 hour post-injection of rHuPH20 or F204P-PH20. By 4 hours post-administration of the enzymes, however, there was not a statistically significant increase in the area of dye dispersion compared to control. In addition, no statistically significant differences in the area of dye dispersion was observed between the rHuPH20 and F204P-PH20 treatment groups. Therefore, the duration of the spreading activity of rHuPH20 and F204P were similar and show that rHuPH20 and F204P-PH20 have comparable in vivo performance.

(616) TABLE-US-00030 TABLE 30 Dermal Reconstitution min time post- Point injection Vehicle rHuPH20 F204P-PH20 30 5 49.96 .+-.± 2.05 80.84 .+-.± 8.03 80.76 .+-.± 4.46 20 64.42

.+-.± 2.49 94.55 .+-.± 7.09 95.75 .+-.± 5.18 1 hour 5 58.01 .+-.± 3.21 82.56 .+-.± 6.40 77.11 .+-.± 3.18 20 65.19 .+-.± 6.21 96.19 .+-.± 6.39 91.45 .+-.± 1.73 4 hour 5 52.10 .+-.± 3.47 67.19 .+-.± 2.39 67.33 .+-.± 3.93 20 57.69 .+-.± 3.92 81.15 .+-.± 4.45 82.21 .+-.± 4.14 24 hour 5 49.87 .+-.± 3.25 59.01 .+-.± 2.15 54.91 .+-.± 3.54 20 57.15 .+-.± 3.47 67.65 .+-.± 2.27 62.91 .+-.± 3.30 48 hour 5 53.64 .+-.± 2.99 53.53 .+-.± 4.88 55.64 .+-.± 7.19 20 61.57 .+-.± 4.02 66.33 .+-.± 4.12 63.11 .+-.± 5.97

Example 16

In Vivo Pharmacokinetics of F204P-PH20 Compared to rHuPH20

[0697](617) The pharmacokinetics (PK) of rHuPH20 and F204P-PH20 were compared following intravenous tail-vein administration by measuring the plasma hyaluronidase levels over time after administration. The proteins used in the study were purified PH20 variant F204P (prepared as described in Example 12; batch concentration 1.02 mg/mL) and wild-type rHuPH20 (prepared as described in Example 1; batch concentration 0.95 mg/mL) formulated in API buffer (10 mM Histidine, 130 mM NaCl, pH 6.5). The proteins were prepared at a concentration of 0.087 mg/mL in API buffer for a dose volume of about 5 mL. An animal that was not administered with protein was used a control (pre-dose control). Forty two (42) male CD-1 mice (.about.~20-30 grams) were used in the study with six animals per treatment group as set forth in Table 31.

(618) TABLE-US-00031 TABLE 31 Pharmacokinetics of Single Intravenous Dose of rHuPH20 or F204P-PH20 Dose Dose number of (mg/Dose Volume Group animals (No.) Test Article (mg/kg) (mL/kg) Euthanasia 1 6 (Nos. 1-6) no treatment N/A N/A pre-dose 2 6 (Nos. 7-12) rHuPH20 0.433 5 ___.sup. 1 min 3 6 (Nos. 13-18) rHuPH20 0.433 5 5 .+-.± 1 min 4 6 (Nos. 19-24) rHuPH20 0.433 5 10 .+-.± 2 min 5 6 (Nos. 25-30) F204P-PH20 0.433 5 ___.sup. 1 min 6 6 (Nos. 21-36) F204P-PH20 0.433 5 5 .+-.± 1 min 7 6 (Nos. 37-42) F204P-PH20 0.433 5 10 .+-.± 2 min

[0698](619) Mice were intravenously administered 0.433 mg/kg rHuPH20 or F204P-PH20 by tail vein injection. Blood samples were obtained from animals 1 minute, 5 minutes and 10 minutes post-administration. Blood samples were obtained by terminal bleed (cardiac puncture) and collected into blood collection tubes containing the anti-coagulant EDTA for the preparation of plasma. Blood samples were centrifuged at 500 g for 10 minutes and the plasma removed and frozen at -80.degree.−80° C. until assessment of hyaluronidase activity using the microturbidity assay described in Example 8.

[0699](620) The results are set forth in Table 32. The results show that hyaluronidase activity is detected in plasma prior to treatment with the hyaluronidase. Within 1 minute post-treatment with either rHuPH20 or F204P-PH20 hyaluronidase, there is a detectably high amount of hyaluronidase activity present in the plasma, which is similar between both treatment groups. Over time, the hyaluronidase activity rapidly decreases for both treatment groups, although there is detectably hyaluronidase activity present in the plasma 10 minutes post-administration. At the 5 minute and 10 minute post-administration time points, activity in the plasma in animals treated with F204P-PH20 is greater than in animals treated with rHuPH20. This shows that F204P-PH20 exhibits somewhat greater activity for a prolonged time period, and therefore exhibits greater half-life in vivo than rHuPH20.

(621) TABLE-US-00032 TABLE 32 rHuPH20 and F204P-PH20 Activity (U/mL) in Mouse Plasma K2EDTA Time Point (min) Predose 1 minute 5 minute 10 minute Animal Animal Animal Animal Protein No. U/mL No. U/mL No. U/mL No. U/mL rHuPH20 1 BQL 7 .sup. 235.sup.a 13 18.3 19 3.76 2 BQL 8 13.5 14 7.70 20 3.70 3 BQL 9 278 15 8.85 21 2.64 4 BQL 10 328 16 10.5 22 2.70 5 BQL 11 356 17 12.8 23 2.36 6 BQL 12 287 18 18.0 24 2.80 F204P-F204P-PH20 1 BQL 25 249 31 48.0 37 11.5 PH20-2 BQL 26 223 32 21.6 38 11.4 3 BQL 27 246 33 38.4 39 10.1 4 BQL 28 246 34 38.6 40 12.2 5 BQL 20 0.696 35 38.2 41 10.8 6 BQL 30 257 36 28.5 42 10.2 BQL—Below Quantifiable Limit <≤0.625 U/mL with minimum required dilution .sup.aHemolyzed

[0700](622) Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claimclaims.

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