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Cloning and characterization of the bovine testicular PH-20 hyaluronidase core domain

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Abstract

The core nucleotide sequence of bovine (*Bos taurus*) testicular PH-20 hyaluronidase was cloned using one step RT-PCR. The 5′ and 3′ regions were cloned separately and a sequence overlap of 124 bp facilitated the fusion of these two fragments by overlapping PCR, resulting in a concatenated sequence of 1422 bp. This nucleotide sequence and its deduced amino acid sequence were compared to homologous sequences from eight other mammal species. The bovine sequences were most similar to those of the pig, *Sus scrofa* (swine Spam1: 79.1% nucleotide and 70.1% amino acid similarity) and least similar to sequences from the Norway rat, *Rattus norvegicus* (murine Spam1: 61% nucleotide and 53.3% amino acid similarity). A phylogenetic analysis joined the red fox (*Vulpes vulpes*) sequence as sister to the bull-pig pair. Twelve cysteine residues were conserved among all nine aligned amino acid sequences and five proposed glycosylation sites have been identified. The feasibility of developing an effective, low-cost bovine PH-20 expression system is discussed in light of these new data.

Introduction

Most vertebrate connective tissue contains hyaluronan, a simple glycosaminoglycan that can be hydrolyzed by a hyaluronidase (Gmachl & Kreil 1993, Hynes & Walton 2000, Zhang & Martin-DeLeon 2001). PH-20 is a testes-specific hyaluronidase that is bound to the acrosomal membrane of the sperm head (Gmachl *et al.* 1993, Hunnicutt *et al.* 1996) and plays a role in fertilization. PH-20 cDNAs from humans and a variety of other mammals have been cloned, characterized and expressed in mammalian cell lines (ten Have *et al.* 1998, Zhang & Martin-DeLeon 2001). In addition, PH-20 active sites have been mapped in humans and crab-eating macaques (*Macaca fasciculata*). These studies reveal that the glycosylphosphatidyl-anchored domain is on the carboxyl terminus (Cherr *et al.* 2001) and that glycosylation occurs during maturation (Li *et al.* 2002). Post-translational proteolytic processing, including disulfide bond formation and modification by a signal peptide, are necessary for the enzyme to attain full activity (Meyer *et al.* 1997, Li *et al.* 2002).

The ability of PH-20 to depolymerize the hyaluronan in connective tissue has been put to a variety of uses, including cancer diagnosis (Godin *et al.* 2000) and inhibition of malignant tumor growth. Topical application can alleviate joint problems, soften dry skin, loosen the foreskin of uncircumcised men and accelerate wound healing after optical surgery (West *et al.* 1997).

This molecule has a number of interesting features and promising applications but a complete understanding of post-translational processing is necessary to gain maximal activity in alternate expression systems. Herein we describe our methods for successfully cloning and characterizing the core nucleotide sequence of bovine testicular PH-20 sequence and its deduced amino acid sequence (GenBank accession number AY297029). We discuss the structure of this gene re-

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gion in relation to designing an inexpensive artificial expression vector for bovine PH-20.

Materials and methods

Isolation of mRNA from bull testes and cDNA synthesis-PCR (RT-PCR)

Total RNA was isolated from testicular tissue using a Qiagen RNeasy kit; mRNA was then isolated using an Oligotex-dT beads (Qiagen). After obtaining a fresh bovine testes from an abattoir in Incheon, South Korea, 60 mg tissue was excised, frozen in liquid N_2 and ground into a powder, 600 μl RLT buffer (RNA extraction buffer containing guanidine isothiocyanate) was added and the mixture was homogenized by passing it through a 23G syringe needle and centrifuged for 5 min. The resulting clear supernatant was mixed with an equal volume of 70% (v/v) ethanol and filtered through an RNeasy column, followed by washing and elution. Total RNA was further isolated using Oligotex-dT resin (Qiagen) as suggested by the manufacturer.

One step RT-PCR was used to synthesize cDNA and amplify the target gene. mRNA, 5 μg, was mixed with 5 × RT-PCR buffer, 400 μM of each dNTP, 20 pmol of the forward degenerate primer [5′-ATGMGBATGYTNMGBMGBCAYCAYAT-3′ (PH20-1)] and 20 pmol of the reverse primer [5′-GGCAGTGCTTTCYTTCCACAACAA-3′ (PH20-4)], then mixed with 1 μl enzyme mixture (MuLV-RT and Taq DNA pol) in a total volume of 25 μl. The PCR was run using the following sequence: 50 °C for 30 min, 94 °C for 15 min, then 35 cycles of [94 °C for 45 s, 45 °C for 45 s, 72 °C for 45 s] and a final 10 min period at 72 °C.

The primers included the 5′ region, the initiation codon and the middle of the sequence. These degenerate primers were designed using the previously characterized amino acid sequence of bovine PH-20 as a guide (Meyer *et al.* 1997). A second pair of primers was then designed to cover the 3′ region [5′-TTATTATCTTTTTCCTGATGTTAC-3′ (PH20-5)] and [5′-GGGAGGTTTTAGAAAAGCATCTATACA-3′ (PH20-Spe)]. Two separate RT-PCR reactions were performed using the different primer pairs.

Cloning, sequencing and joining of full-length PH-20

The expected PCR product sizes of 777 and 775 bp from the 5′ and 3′ regions, respectively, were excised from an agarose gel and purified with a silica membrane spin column (Qiagen). Purified fragments were then ligated into a U-tailed pDrive cloning vector (Qiagen). Clones containing the correct insert were sequenced using an ABI PRISM BigDye Terminators Sequencing Kit. A full-length clone of PH-20 was created by joining the 5′ and 3′ fragments using overlapping PCR. Two separate PCR reactions amplified the 5′ and 3′ PH-20 clones by using Deep Vent DNA polymerase (Biolab) to obtain blunt end PCR products, which were then used as templates in an overlapping reaction. The reaction incorporated primers PH20-1, PH20-Spe and Taq DNA polymerase for direct cloning into a U-tailed vector.

Sequence assembly and analysis

Sequences were analyzed and manipulated with components of the Lasergene 4.03 software package (DNASTAR, Madison, WI; http://www.dnastar.com/). *Seqman* was used to assemble contigs and *MegAlign* was used to compare sequences and assess their similarity. Clustree (http://www1.angis.org.au/WebANGIS/WAG/) was used to construct neighbor joining phylogenetic trees (Saitou & Nei 1987). Predicted signal peptide and cleavage sites were also analyzed based upon the C-score (raw cleavage site score), S-score (signal peptide score) and Y-score (combined cleavage site score) parameters. The cleavage site is presumed to be located where the C-score is high at position +1, the S-score is low at +1 after the spliced site and the Y-score is the highest at +1 (Nielsen *et al.* 1997).

Results and discussion

The nucleotide and deduced amino sequences of bovine PH-20 are shown in Figure 1. There was no internal stop codon nor a frame shift in the nucleotide sequence and 95% of the amino acid sequence was shared with a previous reported bovine PH-20 amino acid sequence that laced corresponding nucleotide sequence information (Meyer*et al.* 1997). The shorter sequence variants also obtained (data not shown) were probably generated from alternative mRNA splicing or from errors generated by the reverse transcriptase encompassing the stem loop of the secondary RNA

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1     ATGGGGATGTTCCGGCGGCATCATATCTCCTTTCGGAGCTTTGCTGGGTCTAGCGGAACACCCCAGGCAGTGTTCACCTT
       M  G  M  F  R  R  H  H  I  S  F  R  S  F  A  G  S  S  G  T  P  Q  A  V  F  T  F
81    CCTTCTGCTTCCGTGTTGTTTGGCTCTGGACTTCAGAGCACCCCCTCTTATTTCAAACACTTCTTTCCTCTGGGCCTGGA
      - L  L  L  P  C  C  L  A /L  D  F  R  A  P  P  L  I  S  N  T  S  F  L  W  A  W -
161   ATGCCCCAGTTGAACGTTGTGTTAACAGAAGATTTCAACTACCTCCAGATCTGAGACTCTTCTCTGTAAAAGGAAGCCCC
      N  A  P  V  E  R  C  V  N  R  R  F  Q  L  P  P  D  L  R  L  F  S  V  K  G  S  P
241   CAGAAAAGTGCTACCGGACAATTTATTACATTATTTTATGCTGATAGACTTGGCTACTATCCTCATATAGATGAAAAAAC
       Q  K  S  A  T  G  Q  F  I  T  L  F  Y  A  D  R  L  G  Y  Y  P  H  I  D  E  K  T
321   AGGCAAAACCGTATTCGGAGGAATTCCCCAGTTGGGAAACTTAAAAAGTCATTTGGAGAAAGCAAAAAATGACATTGCCT
      - G  K  T  V  F  G  G  I  P  Q  L  G  N  L  K  S  H  L  E  K  A  K  N  D  I  A -
400   ATTACATACCAAATGACAGCGTGGGCTTGGCGGTCATTGACTGGGAAAACTGGAGGCCTACCTGGGCAAGAAACTGGAAA
      Y  Y  I  P  N  D  S  V  G  L  A  V  I  D  W  E  N  W  R  P  T  W  A  R  N  W  K
481   CCTAAAGATGTTTACAGGGATGAGTCAGTTGAGTTGGTTCTGCAAAAAAATCCGCAACTCAGTTTCCCAGAGGCTTCCAA
       P  K  D  V  Y  R  D  E  S  V  E  L  V  L  Q  K  N  P  Q  L  S  F  P  E  A  S  K
561   GATTGCAAAAGTGGATTTTGAGACAGCAGGAAAGAGTTTCATGCAAGAGACTTTAAAACTGGGAAAATTACTTCGGCCAA
      - I  A  K  V  D  F  E  T  A  G  K  S  F  M  Q  E  T  L  K  L  G  K  L  L  R  P -
641   ATCACTTATGGGGTTATTATCTTTTTCCTGATTGTTACAATCATAATCATAACCAACCTACTTACAATGGAAATTGCCCT
      N  H  L  W  G  Y  Y  L  F  P  D  C  Y  N  H  N  H  N  Q  P  T  Y  N  G  N  C  P
721   GATGTAGAAAAAAGGAGAAATGATGATCTCGAGTGGTTGTGGAAGGAAAGCACTGCCCTTTTCCCTTCTGTTTATTTGAA
       D  V  E  K  R  R  N  D  D  L  E  W  L  W  K  E  S  T  A  L  F  P  S  V  Y  L  N
800   TATCAGGTTAAAATCTACTCAAAATGCTGCCTTGTATGTTCGTAATCGTGTCCAGGAAGCCATTCGGTTGTCTAAAATAG
      - I  R  L  K  S  T  Q  N  A  A  L  Y  V  R  N  R  V  Q  E  A  I  R  L  S  K  I -
881   CGAGTGTCGAAAGTCCACTTCCGGTTTTTGTATATGCCCGTCCAGTTTTTACTGATGGGTCTTCAACATATCTTTCTCAG
      A  S  V  E  S  P  L  P  V  F  V  Y  A  R  P  V  F  T  D  G  S  S  T  Y  L  S  Q
961   GGTGACCTTGTGAATTCGGTTGGTGAGATCGTTTCTCTAGGTGCCTCTGGGATTATAATGTGGGGCAGTCTCAATCTAAG
       G  D  L  V  N  S  V  G  E  I  V  S  L  G  A  S  G  I  I  M  W  G  S  L  N  L  S
1041  CTTATCTGTGCAATCTTGCATGAACCTAGGCACTTACTTGAACACTACACTGAATCCTTACATAATCAACGTCACCCTAG
      - L  S  V  Q  S  C  M  N  L  G  T  Y  L  N  T  T  L  N  P  Y  I  I  N  V  T  L -
1121  CCGCCAAAATGTGCAGCCAAGTGCTTTGCCATGATGGAGGAGTGTGTACAAGGAAACACTGGAATTCAAGCGACTATCTT
      A  A  K  M  C  S  Q  V  L  C  H  D  G  G  V  C  T  R  K  H  W  N  S  S  D  Y  L
1201  CACCTGAACCCAATGAATTTTGCTATTCAAACTGGGGAAGGTGGAAAATACACAGTACCTGGAACACTGACACTTGAAGA
       H  L  N  P  M  N  F  A  I  Q  T  G  E  G  G  K  Y  T  V  P  G  T  L  T  L  E  D
1281  CCTGCAGAAATTTTCTGATACATTTTATTGCAGCTGTTATAGCAACTTGAGTTGTAAGAAGAGAGTTGATATAAAAAACG
      - L  Q  K  F  S  D  T  F  Y  C  S  C  Y  S  N  L  S  C  K  K  R  V  D  I  K  N -
1361  TTCATTCTGTTGATGTGTGTATGGCTGAAGATGTCTGTATAGATGCTTTTCTAAAACCTCCC
   V  H  S  V  D  V  C  M  A  E  D  V  C  I  D  A  F  L  K  P  P
```

Fig. 1. Partial nucleotide and deduced amino acid sequence of bovine PH-20. The proposed cleavage site of the signal peptide is marked with a slash (/). Two separate clones, 5′ and 3′, were joined by overlapping PCR. The 124 grey-shaded bp denote the overlapping region between the two contigs. A total of 1422 base pairs and 474 amino acid residues were submitted to GenBank (AY297029).

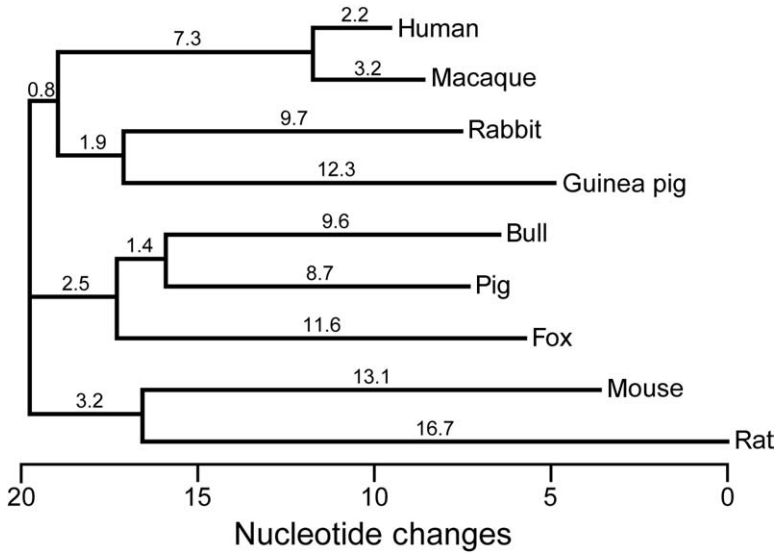

Fig. 2. Neighbor joining phylogenetic tree of the core sperm surface hyaluronidases from nine mammals based on 1409–1421 bp of PH-20 or Spam1. The branch lengths are over the branches. Each core sequence included a signal peptide. Clustree (http://www1.angis.org.au/WebANGIS/WAG/) was used to construct neighbor joining phylogenetic trees. Scientific names are given in Table 1.

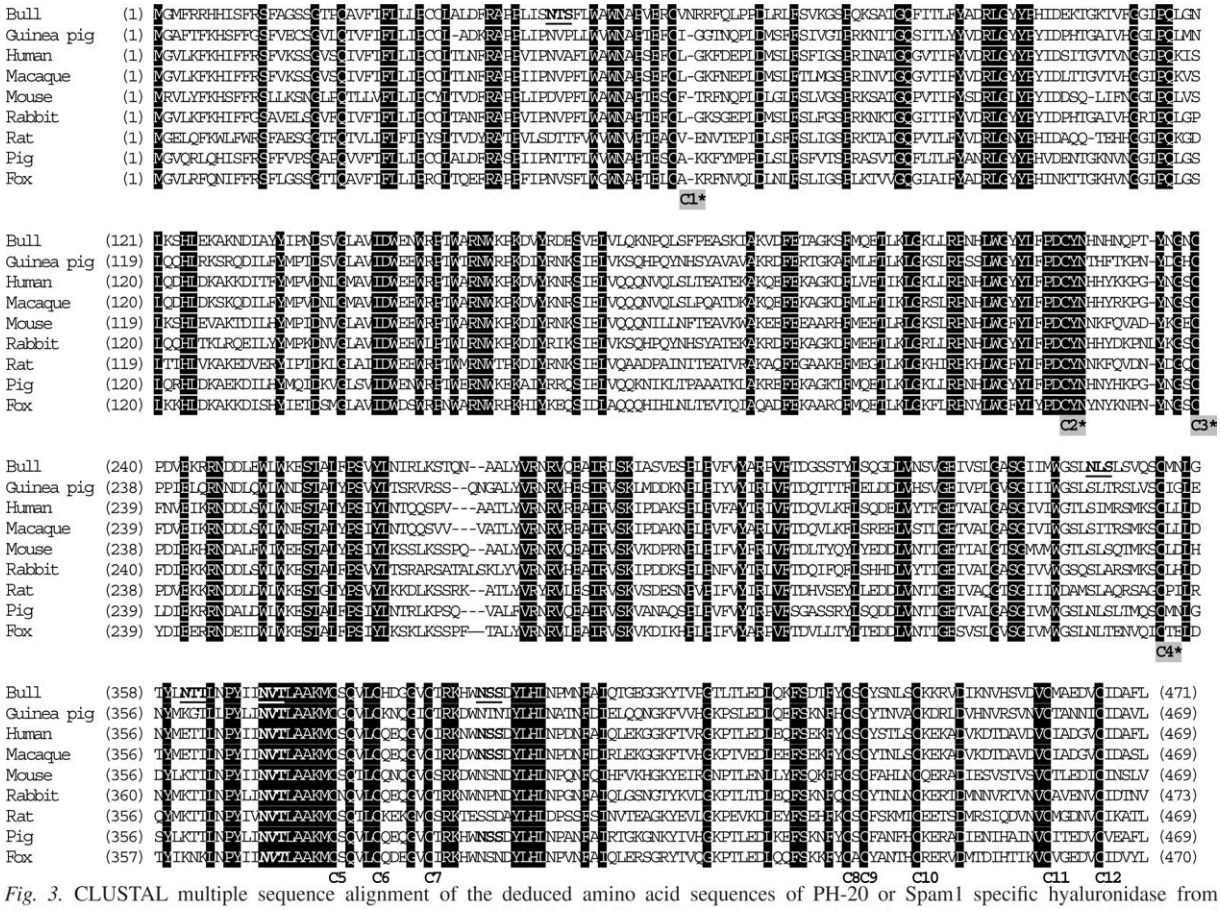

Fig. 3. CLUSTAL multiple sequence alignment of the deduced amino acid sequences of PH-20 or Spam1 specific hyaluronidase from nine mammals. Conserved residues are marked with solid blocks ■, **C1–C12** denote conserved cysteins and proposed bovine (*Bos taurus*) *N*-glycosylation sites are bold and underlined, but note that **NDS** is not glycosylated. The two conserved disulfide bridges are shaded and marked with asterisks: **C1*–C2*** and **C3*–C4***. Scientific names are given in Table 1.

structure. The bovine PH-20 contained a signal peptide that was probably spliced between amino acid residues 35 and 36 (Nielsen *et al.* 1997).

The sequence similarity among bovine PH-20 was compared with eight other homologous mammalian sequences (Table 1) and used these DNA sequence data to generate the gene phylogeny shown in Figure 2. Alignments of these sequences reveal that – apart from a signal peptide – eleven cysteine residues are highly conserved in all nine sequences. Two conserved disulfide bridges (C60-C226 and C240-C354) known from honeybee venom hyaluronidase (*Apis mellifera*, protein.id AAA27730.1, alignment not shown) were found in corresponding locations within the bovine sequence (Gmachl & Kreil 1993).

Two arginine residues are conserved (R283 and R289) suggesting that these residues facilitate substrate binding affinity (Arming *et al.* 1997). We also note that E286 is highly conserved and is surrounded by R283 and R289 adjacent to the active core (Lokeshwar *et al.* 2002). Eight cysteine residues located in the carboxyl terminus are conserved in all sequences spanning C353 to C466 and may be involved in posttranslational protein maturation or activity modulation. Glycosylation is essential for hyaluronidase activity on the recognition motif NxS or NxT (Cherr *et al.* 2001). There are at least five possible *N*-glycosylation sites on bovine PH-20: N45, N345, N361, N370 and N395; this correlates with the previous report of Meyer *et al.* (1997). Of these sites, N_{370}VT is found in all nine sequences (Fiszer-Szafarz *et al.* 2000). The core sequence from 38–376 belongs to glycosyl-hydrolase family. Hyaluronidase possibly contains two functional domains, aa14-172 and aa277-293, which are differentially optimal at neutral and acidic pH, respectively (Cherr *et al.* 2001).

Table 1 Nucleotide and amino acid sequence similarity of PH-20 and its homologues among nine mammalian species. Percent nucleotide similarity is denoted above the diagonal and percent amino acid similarity is below. Sequence data of PH-20 and Spam1 were retrieved from GenBank: **1**) bull, *Bos taurus* PH-20 (AY297029); **2**) Guinea pig, *Cavia cobaya* PH-20 (X56332); **3**) human, *Homo sapiens* PH-20 (S67798); **4**) crab-eating macaque, *Macaca fascicularis* PH-20 (L13780); **5**) house mouse, *Mus musculus* PH-20 (AK017112); **6**) rabbit, *Oryctolagus cuniculus* PH-20 (U09183); **7**) Norway rat, *Rattus norvegicus* Spam1 (NM_053967); **8**) pig, *Sus scrofa* Spam1 (AJ310675); **9**) red fox, *Vulpes vulpes* PH-20 (U41412).

Percent nucleotide sequence identity

1	2	3	4	5	6	7	8	9		Mammal
	66.6	73.7	72	64.9	66.5	61	79.1	73.6	**1**	Bull
58.6		69.8	69.1	63.1	74	60.3	63.5	65.2	**2**	Guinea pig
64	59.5		94.5	66.9	77.2	63.1	75.5	71.5	**3**	Human
62.9	62.7	90.4		65.5	76.1	61.8	73.7	70.5	**4**	Macaque
59.1	56.9	61.4	61.2		64.6	64.5	66.4	65.4	**5**	Mouse
59.7	68.4	70.4	70.4	59.3		61.2	68.5	68.5	**6**	Rabbit
53.3	52.9	54.2	53.7	59.1	53.1		59.6	59.5	**7**	Rat
70.1	55.9	65.9	65	61.2	61.8	51.6		75.2	**8**	Pig
63.8	55	61.8	61.4	61	59.4	53.7	64.2		**9**	Fox

Percent amino acid sequence identity

Successful cloning of the cDNA of bovine PH-20 hinged upon designing primers from the appropriate reference sequence. The degenerate primers were designed based upon previously reported amino acid sequences from bovine PH-20 (Meyer *et al.* 1997). The bovine nucleotide sequence was not available from GenBank, but a partial cDNA sequence in the 3′ region of ovine PH-20 (*Ovis aries*, sheep) was retrieved (AF174691). When aligned with eight other mammalian PH-20 sequences and their homologues (Table 1 and Figure 3), the bovine sequence was most similar to that of the pig, *Sus scrofa*. An intact core domain of bovine PH-20 was successfully obtained and deposited in GenBank (AY297029). The domain shared several features with mammalian PH-20 and Spam1, human Hyal1 and honeybee venom hyaluronidase.

Testicular hyaluronidase has been used in the cosmetic, pharmaceutical and medical fields, and shows promise as a diagnostic tool for screening cancer patients. However, the advent of bovine spongiform encephalitis (mad cow) diseases halted commercial production of bovine PH-20, which was purified from bull testes. To fill this need, several attempts have been made to produce PH-20 in animal cell culture (Gmachl *et al.* 1993, Seaton *et al.* 2000). Honeybee venom hyaluronidase has been expressed in the bacterium *Escherichia coli* but its activity was relatively low (3–20%) when compared with the native product or to a recombinant enzyme produced by insect cells infected with recombinant baculovirus.

PH-20 requires glycosylation and other posttranslational modifications to attain full activity. While *E. coli* and other bacteria lack the capacity to glycosylate proteins, yeasts possess the necessary molecular mechanisms. The yeast, *Saccharomyces cerevisiae*, has been used as an expression vector for proteins requiring glycosylation, but it often does the job too well: most proteins expressed by *S. cerevisiae* are too heavily glycosylated with mannose and therefore lose some of their functional activity (Schuster *et al.* 2000). However, proteins expressed in the yeast *Pichia pastoris* show intermediate glycosylation levels and this species may therefore offer a suitable PH-20 expression system.

Complete understanding of proteolytic cleavage is an essential step in the refinement of *P. pastoris* as a PH-20 expression vector. After cleavage, the *C*-terminus of the PH-20 and Hyal1 pre-proteins leave an oligopeptide attached to the domain by a disulfide

bridge. There is no indication that this small peptide plays any role in posttranslational modification, nor that it serves as an anchor for attachment, but these possibilities must be investigated (Cherr *et al.* 2001). The ideal expression vector would contain a secretory signal peptide to facilitate movement of the expressed protein into media (Verkhusha *et al.* 2003). An endo-β-1,4-glucanase from the edible straw mushroom (*Volvariella volvacea*) expressed in *P. pastoris* has shown relatively high activity, even though glycosylation levels were higher than in the native enzyme (Ding *et al.* 2002).

The core domain of bovine PH-20 (excluded signal peptide) was successfully subcloned into a pPIC9 expression vector (Invitrogen, http://www.ivitrogen.com) containing α-factor secretion signal and subsequently transformed into *P. pastoris* GS115 host strain (data not shown). These clones are being optimized and optimized to maximize PH-20 expression into culture media. Yeasts are not the only expression system amenable to PH-20 production but, because of their ease-of-use and cost effectiveness, merit first priority in investigation of recombinant glycosylated PH-20 production. Plant liquid cell culture suspension is another inexpensive means by which a gene of interest can be inserted into a plant virus potyviral genome, such as potyvirus Y (PVY). The protein of interest will be processed by viral proteinase and is subsequently purified by means of metal chelating affinity resin (Afaq & Iqbal 2001).

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