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Review

Hyaluronidases, a group of glycosidases: Current and future perspectives

Nermeen S. El-Safory[a], Ahmed E. Fazary[b,c], Cheng-Kang Lee[a,*]

[a] Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 106-07, Taiwan, ROC
[b] Egyptian Organization for Biological Products and Vaccines (VACSERA Holding Company), 51 Wezaret El Zeraa St., Agouza, Giza, Egypt
[c] School of Pharmacy, College of Medicine, National Taiwan University, Taipei 100, Taiwan

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ABSTRACT

Given the constant synthesis and degradation of hyaluronan (hyaluronic acid, HA) in tissues, it is remarkable that the human body maintains precise levels of hyaluronan as tightly as it does. Hyaluronidases represent a group of glycosidases, which mainly degrade hyaluronan, a linear, non-sulfated polysaccharide composed of repeating disaccharide units [D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]n. They are widely distributed in nature, being found in mammals, insects, leeches and bacteria. There has been a plethora of review articles on hyaluronidases, either as a subject on their own or as part of a review on glycosidases enzymes. The present review summarizes the current research on their classification, sources, activity assays, bio-physical and chemical properties, crystal structural features and its catalytic mechanism. Special emphasis is given to the importance role of that type of enzymes in biotechnological processes, as well as its medicinal and bio-industrial applications.

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* Corresponding author. Tel.: +886 2 2737 6629; fax: +886 2 2737 6644.
E-mail address: cklee@mail.ntust.edu.tw (C.-K. Lee).

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1. Introduction

Hyaluronidases (Hyals) are classes of glycosidases that predominately degrade hyaluronic acid (HA), with limited ability to degrade chondroitin and chondroitin sulphates (Table 1). Also, hyaluronidases are endoglycosidases, as they can degrade the β-N-acetyl-D-glucosaminidic linkages in the HA polymer. Since it was known in the first half of the last century, hyaluronidases have become with considerable roles in biotechnological processes mainly in pharmaceutical industry. Therefore, the discovery of new hyaluronidases with novel properties continues to be an important research area. The amount of research work directed towards hyaluronidases has increased dramatically since 1940. For example, over the periods 1940–1960 and 1960–present, the average number of refereed publications describing research involving hyaluronidases as a major component (within abstract, title, and keywords; using Scopus search engine) was 420 and 8004, respectively. In the last decade (2000–2009), it is interesting to note that the publications concerned with hyaluronidases observed between 2000 (201 documents) and 2009 (239 documents) coincides with the most recent discoveries on isolation, purification, characterization and applications of hyaluronidases. There were also striking increases in the number of publications concerned with hyaluronic acid (the related compound to hyaluronidases). The average number of documents was 92 for the period (1940–1960), while for the period 1960–present, the average number was 21684 using the same search engine (Scopus). Among all of this research work, a very few reports were found in literature describe concisely hyaluronidases types. In this review, we concentrate on the previous relevant studies conducted in hyaluronidases sources, their production, bio-physico-chemical properties, crystal structural features, and finally their biological functions.

Hyaluronan (hyaluronic acid, or hyaluronate, HA) (Fig. 1) is a high-molecular mass polysaccharide found in the extracellular matrix, especially of soft connective tissues. Commonly, hyaluronan is known as a lubricant responsible for the viscoelastic properties of tissue fluids and as a stabilizing and hydrating component of soft connective tissue. The chemical structure of hyaluronic acid of repeating disaccharide units linked by β-1,4 glycosidic bonds has been elucidated in which each disaccharide unit consists of D-glucuronic acid (GlcUAc) and N-acetyl-D-glucosamine (GlcNAc) connected by a β-1,3 glycosidic bond. Depending on the tissue source, this polymer usually consists of 2000–25000 disaccharides, giving rise to molecular masses ranging from 10^6 to 10^7 Da with extended lengths of 2–25 μm. Under *in vivo* conditions hyaluronic acid exists as a polyanion as the carboxyl groups of the glucuronic acid ause the high negative charge of the polymer under physiological conditions. In other words, hyaluronic acid is a high-molecular weight, highly anionic glycosaminoglycans (GAG) (Fig. 1), including chondroitin-, dermatan- and keratan sulphate, heparin and heparan sulphate.

The described secondary structure of HA allows for a further organization of the macromolecule in an aqueous environment and its tertiary structures are specifically and reversibly disaggregated by mild physicochemical methods (Scott, Cummings, Brass, & Chen, 1991; Scott & Heatley, 2002). This dynamic meshwork provides the basis for the viscoelastic and hydrating properties of hyaluronan. In solutions, between the HA chains a huge amount of water can be trapped, which can be withdrawn upon application of an external pressure causing the resilience and malleability of substances like synovial joint fluids (Toole, 2004; Prehm, 1984, 1990, 2006). In addition, the hydrophobic interactions and hydrogen bonds that can form between HA chains are counteracted by electrostatic repulsion of the carboxyl groups (Scott, Heatley, & Hull, 1984; Heatley & Scott, 1988; Heldin & Pertoft, 1993). Thus, HA is able to form electrostatic complexes with many proteins, including hyaluronidases (Vincent & Lenormand, 2009). Under the physiological conditions, the supramolecular organization of HA was found to be on the edge of stability, suggesting that reversible formation and breakdown of tertiary structures control important biological properties (Toole, 2004; Scott & Heatley, 1999).

Hyaluronan is found in the extracellular matrix of all vertebrates and in the capsule of some bacteria (Stern, 2004; Prehm, 1984, 1990, 2006; He et al., 2009). There are three reports (Laurent & Fraser, 1992; Necas, Brauner, & Kolar, 2008; Stern, 2004) describe metabolisms, physiological and pathological functions, basic pharmacological properties, and the clinical use of hyaluronic acid. The functions of hyaluronan directly originating from its physicochemical properties are well-known: it acts as a lubricant and shock absorber, regulates water balance and osmotic pressure and occurs as a structure forming molecule in the vitreous humor of the eye, in Wharton's jelly and in joint fluids. Hyaluronan fulfils several distinct physiological functions that contribute both to structural properties of tissues and to cell behaviour during formation or remodelling of tissues (Knudson, Bartnik, & Knudson, 1993). In addition to the functions arising directly from the physicochemical properties of the polymer, hyaluronan also exerts biological effects via specific interactions with hyaluronan-binding proteins (hyaladherins) (El Maradny et al., 1997; Evanko & Wight, 1999). The great number of hyaladherins known so far can be grouped into (i) the structural hyaluronan-binding proteins of the extracellular matrix, such as link protein and the aggregating proteoglycans, (ii) cell surface hyaluronan receptors and (iii) intracellular hyaluronan-binding proteins (Feinberg & Beebe, 1983; Prehm, 2002).

2. Classification of hyaluronidases

Molecular genetic analysis has shown that, hyaluronidases can be grouped according to amino acid sequence homology (Csoka, Frost, Wong, & Stern, 1997; Csoka, Frost, & Stern, 1997; Csoka, Frost, & Stern, 2001) into two main families: the hyaluronidases from eukaryotes and from prokaryotes. The first classification of hyaluronidases was established according to their catalytic mechanism into three main families (Meyer, 1971). This classification was based on substrate specificity and on biochemical analysis of the hyaluronidases and their reaction products (Fig. 2).

The first group of hyaluronidases are the hyaluronate 4-glycanohydrolases, mammalian hyaluronidases (EC 3.2.1.35) which degrading HA by cleavage of the β-1,4-glycosidic bond furnishing tetrasaccharide molecule as the main product. These enzymes are glycosidases with both hydrolytic and transglycosidase activity and degrade HA, chondroitin, chondroitin-4, 6-sulphate to a small extent, dermatan sulphate. The best known enzymes of this class are testicular, lysosomal and bee venom hyaluronidase.

Table 1
Degradative enzymes.

Enzyme	EC No.	Organism	Reaction	Role in pathogenesis	Reference
Collagenase	EC 3.4.24.3	*C. perfringens*	Degrades collagen	Spread of organism in tissue planes	Birkedal-Hansen (1987)
Hyaluronidase	EC 4.2.2.1	*S. pyogenes*	Degrades connective tissue	Spread within tissue planes	Stern and Jedrzejas (2006)
Streptokinase	EC 3.4.99.0	*S. pyogenes*	Indirectly activates plasmin, which degrades fibrin clots	Facilitates spread in vascular and soft tissues	McArthur et al. (2008)
Phospholipase	EC 3.1.4.3	*C. perfringens*	Removes charged head group from phospholipid	Host cell damage	Takahashi, Sugahara, & Ohsaka (1974)

The second group is represented by hyaluronidases occurring in the salivary glands of leeches and hookworms. These enzymes are hyaluronate 3-glycanohydrolases, hyase from leech (EC 3.2.1.36) which degrade HA by cleavage of the β-1,3-glycosidic bond, thus, yielding sugar fragments having glucuronic acid at the reducing end. These enzymes generate tetra- and hexasaccharide end products.

The third group, the microbial hyaluronidases, (bacterial lyases (EC 4.2.2.1)) is hyaluronate lyases. They degrade HA by a β-elimination reaction to yield unsaturated disaccharide 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose as the main product. The hyaluronate lyases, were isolated from various microorgansims, including e.g. strains of *Clostridium, Micrococcus, Streptococcus,* and *Streptomyces*, that differ in substrate specificity (Suzuki, Terasaki, &Uyeda, 2002; Abramson, 1973; Pritchard, Lin, Willingham, & Baker, 1994).

3. Sources of hyaluronidases

Hyaluronidases are widely distributed in nature, being found in mammals, invertebrate animals (crustaceans, leeches, and insects), pathogenic fungi (*Candida*, *Streptomyces*), bacteria and bacteriophage (Kreil, 1995) (Table 2). The variety of hyaluronidases activity in different mammalian tissues was demonstrated (Bollet, Bonner, & Nance, 1963). It was found in testes (Senn, Germond, & De Grandi, 1992) and in various somatic tissues, e.g. liver, kidney, lymphatic system and skin. Also hyaluronidases were found in animal's venoms (Tan & Ponnudurai, 1992a,b), e.g. snakes, lizards, scorpion, stonefish, social wasp and bee venom. A wide variety of microorganisms produce enzymes capable of degrading hyaluronate (Table 2). To date, the amino acid sequences of a variety of hyaluronidases from prokaryotes have been decoded (Suzuki et al., 2002; Hynes & Walton, 2000). The best known and characterized bacterial hyaluronidases are the hyaluronate lyases from *Streptococcus pneumoniae* and *Streptococcus agalactiae* (Jedrzejas & Chantalat, 2000; Pritchard et al., 2000; Jedrzejas, Mello, de Groot, & Li, 2002).

4. Property, crystal structural features and catalytic mechanisim of hyaluronidases

Mammalian hyaluronidases (E.C. 3.2.1.35) belong, according to the Carbohydrate Active Enzyme Database (CAZY), to the glyco-

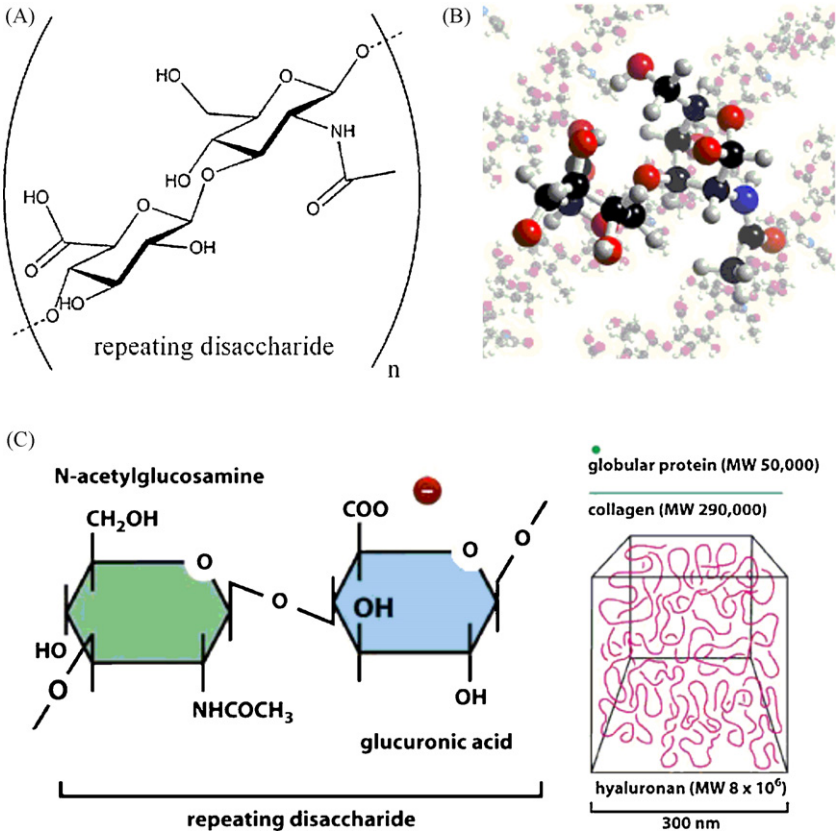

Fig. 1. Structure of hyaluronan. (A) Chemical structure of hyaluronan, the polymer is built of alternating units of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNac). All the glycosidic linkages are β type, 1–3 glycosidic bonds between GlcUA and GlcNac, and 1–4 bonds between GlcNac and GlcUA. (B) 3D model of the hyaluronan structure. (C) Hyaluronan is abundant, long, unsulfated GAG (up to 25,000 sugars), not covalently linked to a core protein and not secreted, but it is synthesized in the ECM by an enzyme complex in the plasma membrane (Scott et al., 1991; Scott and Heatley, 2002).

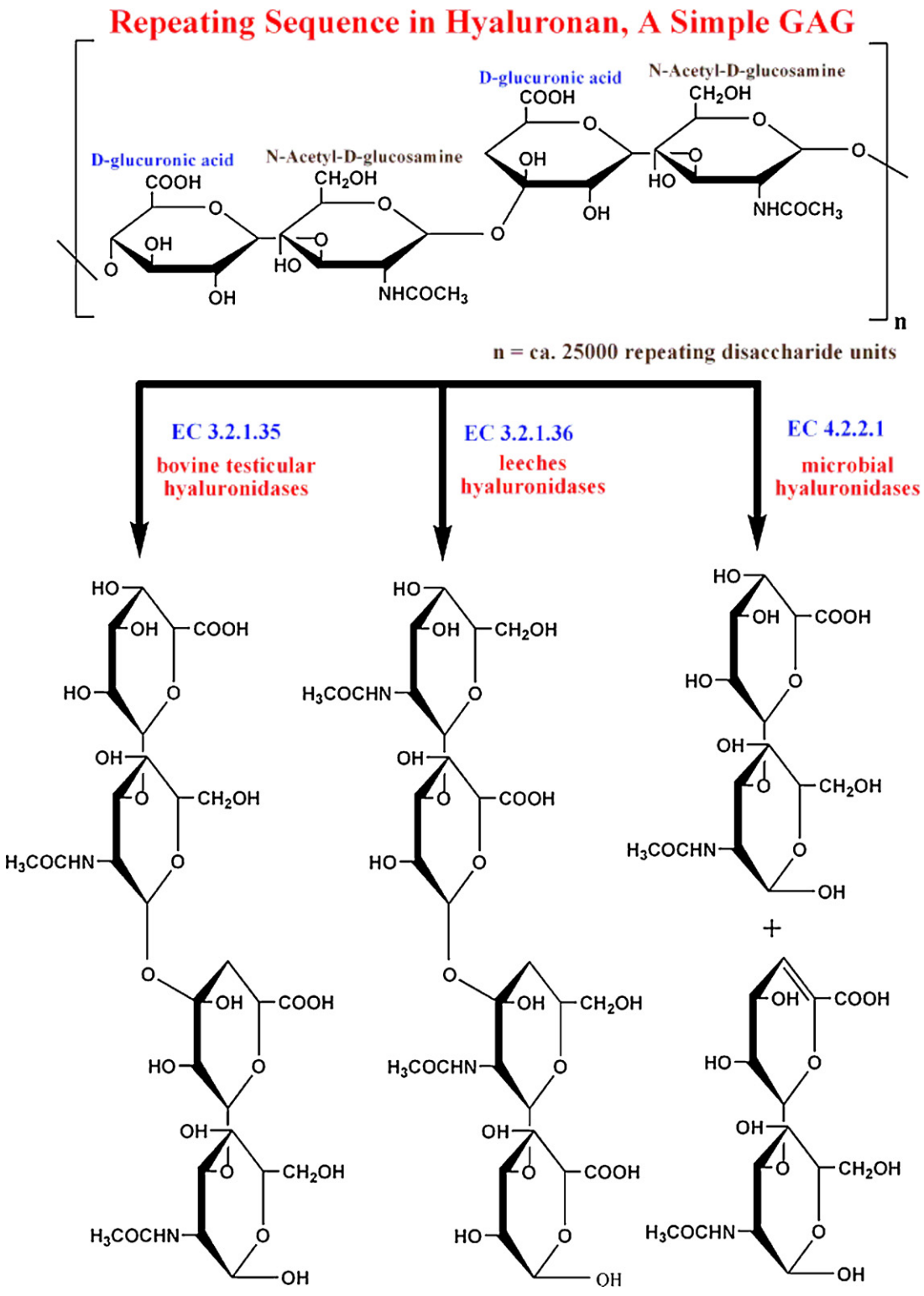

Fig. 2. Classification of hyaluronidases (Meyer, 1971).

side hydrolase family 56. They cleave the β-1,4 glycosidic bond of hyaluronic acid by hydrolysis in a random, nonprocessive endolytic process producing tetrasaccharides as the major final reaction products (Stern & Jedrzejas, 2006). The process of degradation differs essentially between mammalian hyaluronidases and bacterial hyaluronate lyases. They belong to a different CAZY family (polysaccharide lyase family 8). Lyases degrade HA after an initial endolytic cleavage step in a processive, exolytic fashion, thereby separating only one disaccharide unit from the reducing end of the HA chain at a time (Li, Kelly, Lamani, Ferraroni, & Jedrzejas, 2000; Li & Jedrzejas, 2001). The degradation of HA by mammalian hyaluronidases, however, occurs in a random fashion with each endolytic cleavage step being accompanied by a new binding event at a random position within the HA chain (Stern & Jedrzejas, 2006). In addition to the degradation of HA, vertebrate-like hyaluronidases are capable of catabolizing chondroitin, chondroitin-4-sulfate, chondroitin-6-

Table 2
Sources of hyaluronidases.

No.	Organism	Production and purification conditions	Activity (U/mg)	References
EC 3.2.1.35 – hyaluronoglucosaminidase				
1	*Palamneus gravimanus*	25.6-fold to homogeneity from venom by gel filtration and ion exchange chromatography	6412	Morey et al. (2006)
2	*Ovis aries aries*	Partially purified	7.47	Morton (1973)
3	*Hippasa partita*	Venom gland extract, at 37 °C, Sephadex G-100 column chromatography	0.03767	Nagaraju, Devaraja, and Kemparaju (2007)
4	*Hippasa partita*	After 20.4-fold purification, at 37 °C, CM-Sephadex C-25 column chromatography	0.00184	Nagaraju et al. (2007)
5	*Apis mellifera*	Recombinant enzyme expressed in Pichia pastoris strain GS115, HiTrap HP SP column chromatography	0.000239	Reitinger, Mullegger, and Lepperdinger (2001)
EC 3.2.1.36 – hyaluronoglucuronidase				
6	*Hirudo medicinalis*	Leeches were frozen at −70 °C and cut into a head fraction and a crop fraction. Each fraction was ground with sand by pestle and mortar in 0.15 M NaCl and extracted overnight in the cold. These samples were centrifuged (1500 × *g* for 15 min) and the supernatant fluid was brought to 80% ammonium sulphate and equilibrated overnight. These samples were then centrifuged (1500 × *g* for 15 min) and the precipitate was taken up in 0.1 M NaAcetate, pH 6.0, dialyzed overnight against water in the cold, and lyophilized	5000, 42.05, 0.201	Hovingh and Linker (1999)
7	*Nephelopsis obscura*		0.722, 0.325	Hovingh and Linker (1999)
8	*Erpobdella punctata*		0.278, 0.205	Hovingh and Linker (1999)
9	*Desserobdella picta*		0.117, 0.111	Hovingh and Linker (1999)
10	*Placobdella ornata*		0.102, 0.029	Hovingh and Linker (1999)
11	*Theromyzon sp.*		0.09	Hovingh and Linker (1999)
12	*Glossiphonia complanata*		0.058, 0.028	Hovingh and Linker (1999)
13	*Helobdella stagnalis*		0.055, 0.032	Hovingh and Linker (1999)
14	*Haemopis marmorata*		0.038, 0.027	Hovingh and Linker (1999)
EC 4.2.2.1 – hyaluronate lyase				
15	*Streptococcus agalactiae*	Purified 111 and 92 kDa pro-form	3680, 3571	
16	*Streptococcus pyogenes bacteriophage H4489A*	Expression in *Escherichia coli*, pH 5.0: about 40% of maximal activity, pH 9.0: about 55% of maximal activity	9.46	Yang and Lee (2006)
17	*Streptococcus pneumoniae*	Wild-type enzyme, in absence of NaCl and mutant F343V, in absence of NaCl, mutant W292A, in absence of NaCl and mutant W292A/F343V, in absence of NaCl	231.4, 68.9, 4.3, 0.19	Nukui, Taylor, McPherson, Shigenager, & Jedrzejas (2003)
18	*Propionibacterium acnes*	Gel filtration of 85.11 kDa form	7.75	Ingham, Holland, Gowland, & Cunliffe (1979)

sulfate and dermatan sulfate as alternative substrates (Asteriou, Vincent, Tranchepain, & Deschrevel, 2006). The conversion of these alternative substrates, however, is much slower than the degradation of HA. Furthermore, mammalian hyaluronidases exhibit not only hydrolytic activity but also transglycosidase activity. During transglycosylation a HA fragment of at least one disaccharide unit is transferred from the non-reducing end of one oligosaccharide chain to the non-reducing end of another (Highsmith, Garvin, & Chipman, 1975; Takagaki et al., 1994; Takagaki, Nakamura, Izumi, Saitoh, & Endo, 1994).

4.1. Eukarytes hyaluronidases

4.1.1. Human hyaluronidases

Based on the gene sequence, human genome consists of six hyaluronidase genes, clustered into two groups. Three genes are found tightly clustered on chromosome 3p21.3 and coding for HYAL-1, HYAL-2 and HYAL-3. While, another three genes HYAL-4, HYAL-P1 (a pseudo gene), and PH-20/SPAM1 are clustered on chromosome 7q31.3 (Csoka et al., 1997a,b; Csoka et al. 1998; Csoka, Scherer, & Stern, 1999; Csoka et al. 2001). HYAL-P1 is expressed pseudo gene no protein translates. The sequence similarity between the six subtypes of human hyaluronidases varies between 33 and 42% (Stern & Jedrzejas, 2006). The extensive homology between the different hyaluronidase genes suggests ancient gene duplication.

Hyal-1, first isolated from human plasma, is the predominant hyaluronidase in mammalian plasma and urine, and is also found at high levels in the liver, kidney, spleen and heart. It is localised in lysosomes and active at acidic pH (Frost, Csoka, Wong, & Stern, 1997; Frost & Stern, 1997). Also the Hyal-2 protein was found in many tissues, except the adult brain (Lepperdinger, Strobl, & Kreil, 1998). It is localised either in lysosomes or anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) link.

Both Hyal-1 and Hyal-2 are expressed in the somatic tissues such as liver, kidney, spleen etc., and are responsible for the catabolism of intracellular and extracellular HA, respectively (Stern & Jedrzejas, 2006; Csoka et al., 1999). The expression of these enzymes is extremely low and difficult to purify. Recent development in Hyase detection procedure (substrate gel assay) (Guntenhoner, Pogrel, & Stern, 1992) has facilitated the purification of 57 kDa molecular weight Hyal-1 to homogeneity from human plasma (Guntenhoner et al., 1992) (Table 3). In contrast to plasma one, urine contains two hyaluronidases with molecular weight of 57 and 45 kDa. Micro sequencing confirmed that both urinary isozymes have N-terminal identical to plasma hyaluronidase. Lower molecular weight isozyme contains a second N-terminal sequence, which was derived from the C-terminal end of the protein. This suggests that 45 kDa isozyme, resulting from endoproteolytic cleavage of the 57 kDa isoform, consists of two polypeptides linked by disulfide bond (Guntenhoner et al., 1992). In vertebrates, the turnover of HA is controlled by hyaluronidase, which is an endoglycosidase that

Table 3
Bio-physico-chemical properties of hyaluronidases enzymes.

No.	Organism	MW (kDa)	Isoelectric point (pI)	pH optimum	pH stability	Temp optimum	Tempre stability	Reference
Hyaluronoglucosaminidase (EC. 3.2.1.35)								
1	*Naja naja*	54, 70.41	9.7, 9.2	5	–	37 °C	–	Girish and Kemparaju (2005a,b), Girish, Shashidharamurthy, Nagaraju, Gowda, and Kemparaju (2004)
2	*Macaca sp.*	–	5.1	7	–	37 °C	–	Li, Yudin, Robertson, Cherr, and Overstreet (2002)
3	*Nephrops norvegicus*	320	–	5.4	–	45		Krishnapillai et al. (1999a,b)
4	*Homo sapiens*	59, 47, 50, 45, 48, 50	–	5, 4.9, 4, 5.6, 3.5	3.3, 3.7	37 °C	–	Afify, Stern, Guntenhöner, Stern (1993), Toida, Ogita, Suzuki, Toyoda, and Imanari (1999), Maingonnat et al. (1998); Schwartz, Shuster, Jumper, Chang, and Stern (1996), Vigdorovich et al. (2007), Chao et al. (2007), Christopoulos et al. (2006), Hofinger et al. (2007)
5	*Mus musculus*	68	–	–	–	–	–	Thaler and Cardullo (1995)
6	*Streptococcus agalactiae*	116	–	6.3	–	–	–	Ozegowski, Gunther, and Reichardt (1994)
7	*Synanceja horrida*	62	–	6	5–9	37 °C	26–55 °C	Poh et al. (1992)
8	*Streptococcus dysgalactiae*	55	–	5.6	–	40 °C	–	Sting, Schaufuss, and Blobel (1990)
9	*Streptococcus equinus*	55	–	5.6	–	40 °C	–	Sting et al. (1990)
10	*Streptococcus zooepidemicus*	55	–	5.6		40 °C	–	Sting et al. (1990)
11	*Heterometrus fulvipes*	82	–	4	4–6	10 °C	–	Ramanaiah, Parthasarathy, and Venkaiah (1990)
12	*Bos taurus*		–	6.5		22 °C	–	Okorukwu and Vercruysse (2003)
13	*Hippasa partita*	42.26		5.8		37 °C		Nagaraju et al. (2007)
14	*Loxosceles intermedia*		–	7	6–8	–	–	Da Silveira et al. (2007)
15	*Sus scrofa*	55, 65, 70, 80	–	–	4–8, 6–8	–	–	Cibulkova, Manaskova, Jonakova, and Ticha, 2007
16	*Apis mellifera*	–	–	–	3–8	90 °C	4–90 °C	Reitinger et al. (2008)
17	*jaagsiekte sheep retrovirus*	–	–	5.5	3.8–11	37 °C	–	Vigdorovich, Miller, and Strong (2005)
18	*Bos taurus*	–	–	3.6	–	37 °C	–	Salmen et al. (2005)
19	*Apis mellifera*	–	–	3.6	–	37 °C	–	Salmen et al. (2005)
20	*Palamneus gravimanus*	52	–	4.5	–	37 °C	30–40 °C	Morey, Kiran, and Gadag (2006)
21	*Mus musculus*	–	–	5	–	–	–	Hemming et al. (2008)
Hyaluronoglucuronidase (EC 3.2.1.36)								
1	*Bos taurus*	–	–	6.3	–	37	–	Asteriou et al. (2001)
Hyaluronate lyase (EC 4.2.2.1)								
1	*Streptococcus equisimilis*	90	–	–	–	–	–	Ozegowski, Gerlach, and Köhler (1981)
2	*Propionibacterium acnes*	85.11	–	6.4	4.2–7	50 °C	–	Ingham et al. (1979)
3	*Streptococcus sp.*	70	–	6	5.5–8.6	–	20 °C	Hill (1976)

4	*Staphylococcus aureus*	84	–	4.8, 6, 8.6, 5.3	4.6–7	–	20, 37, 56, 4 °C	Rautela and Abramson (1973), Vesterberg (1968), Abramson and Friedman (1968), Arvidson (1983), Makris, Wright, Ingham, and Holland (2004)
5	*Streptococcus pyogenes*	70	–	5.9	5–8	45 °C	–	Gerlach and Köhler (1972)
6	*Streptococcus pneumoniae*	82.28	–	6	–	22, 37, 25 °C	55 °C	Jedrzejas, Mewbourne, Chantalat, and McPherson (1998), Kelly, Taylor, Li, and Jedrzejas (2001), Li, Taylor, Kelly, and Jedrzejas (2001), Akhtar and Bhakuni (2003, 2007), Nukui, Taylor, McPherson, Shigenaga, and Jedrzejas (2003)
7	*Streptococcus dysgalactiae*	–	–	6.2	–	37 °C	–	Suzuki et al. (2002)
8	*Streptomyces hyalurolytis*	–	–	6.2,6 6.5	–	60, 22, 37 °C	–	Suzuki et al. (2002), Okorukwu and Vercruysse (2003)
9	*Streptococcus agalactiae*	92, 111	–	5, 6.8, 6	–	37, 30, 25 °C	–	Jedrzejas et al. (2000); Botzki et al. (2005), Salmen et al. (2005), Pritchard et al. (2000), Kuehn, Ozegowski, Peschel, and Neubert (2004)
10	*Streptococcus pyogenes bacteriophage H4489A*	–	4.7	6	5–9	30 °C	–	Baker, Dong, and Pritchard (2002), Akhtar, Krishnan, and Bhakuni (2006), Yang and Lee (2006)
11	*Streptococcus pyogenes phage H10403*	110	–	6	–	25 °C	–	Mishra, Akhtar, and Bhakuni (2006)
12	*Homo sapiens*	–	–	6	5.5–7.5	–	–	Harada and Takahashi (2007)
13	*Streptococcus suis*	126.5	–	5.5	–	37 °C	–	Allen et al. (2004)

acts jointly with two lysosomal exoglycosidases, β-glucuronidase and β-N-acetyl glucosaminidase.

Like Hyal-1, the Hyal-2 enzyme has a pronounced activity optimum at pH 4; however, as shown for Hyal-2 from *Xenopus laevis*, low activity can be also detected under physiological conditions. Hyal-2 has an unusual substrate specificity cleaving high-molecular mass HA to intermediate size fragments of approximately 20 kDa (50–60 disaccharide units) (Lepperdinger, Müllegger, & Kreil, 2001). As the major hyaluronidases in mammalian somatic tissues Hyal-1 and Hyal-2 are believed to act in succession on degrading HA: HA fragments of approx. 20 kDa are generated at the cell surface by GPI-anchored Hyal-2, internalised and then further digested to tetrasaccharides by the lysosomal Hyal-1 (Csoka et al., 2001; Stern, 2004).

Very little is known about the Hyal-3 protein, but no activity can be identified using the available hyaluronidase assays (Nagata et al., 2004). It was found to be expressed ubiquitously with extraordinarily high expression patterns detected in mammalian testis and bone marrow. The stem-cell like state of these cell types indicates a role of Hyal-3 in embryogenesis and stem-cell regulation (Csoka et al., 2001). The catalytic properties of Hyal-3 are currently unknown (Stern & Jedrzejas, 2006).

Hyal-4 is probably a GPI-anchored protein like PH-20 and Hyal-2 (Lin, Mahan, Lathrop, Myles, & Primakoff, 1994; Prehm, 1984) and is exclusively expressed in muscle and placenta. It was proposed to be the first vertebrate chondroitinase with no activity against HA (Csoka et al., 2001; Rigden & Jedrzejas, 2003). This substrate specificity is in marked contrast to the Hyal-1 and PH-20 enzymes, which can cleave both HA and – at a slower rate – chondroitin sulphate. However, experimental evidence is still lacking, and the only hint for a specialized function of Hyal-4 is the presence of a C residue (C163) in a position close to the active site and strictly conserved in other hyaluronidases.

The pseudogene, HYAL-P1, is transcribed, yet the mRNA contains an internal stop codon resulting in preliminary abortion of translation. However, translation of HYAL-P1 into an active enzyme might occur in other species. Furthermore, MGEA5, a cytoplasmic β-N-acetylglucosaminidase, was described to exhibit an additional hyaluronidase activity (Comtesse, Maldener, & Meese, 2001a,b). However, no sequence similarity could be found between the six human hyaluronidases and MGEA5.

The PH-20 protein (SPAM 1 (sperm adhesion molecule 1)) appears to be a multifunctional protein protein in a membrane-anchored and in a soluble isoform (Cherr, Yudin, & Overstreet, 2001). The GPI-anchored protein is located on the surface of mammalian sperm and in the lysosome derived acrosome, where it is linked to the inner acrosomal membrane. The major role of PH-20 is to facilitate penetration of the sperm through the HA-rich matrix of the oocyte. It is relatively specific for testes, however, by sensitive techniques, it can also be detected in the epididymis, the female genital tract, breast, placenta and fetal tissues. Also in certain malignancies the expression of PH-20 is reported.

While plasma membrane PH-20 shows hyaluronidase activity only at neutral pH, inner acrosomal membrane PH-20 was found to be active both at neutral and acidic pH (Table 3). Recent results (Yudin, Li, Robertson, Cherr, & Overstreet, 2001) suggest that the enzyme activities at different pH involve two different domains in the protein: PH-20 may attain the capability of acid-active hyaluronidase activity after the acrosome reaction, where the enzyme is endoproteolytically cleaved but held together by a disulfide bond. This endoproteolysis may alter the three-dimensional structure of PH-20, enabling the domain, which is responsible for activity at acidic pH, to become active and thus imparting both neutral and acid-active activities to PH-20 (Cherr et al., 2001, 1996).

4.1.2. Bovin testicular hyaluronidases (BTH)

It has been known for a long time that extracts from mammalian testes contain hyaluronidase activity (Chain & Duthie, 1939), and preparations of bovine and ovine testicular hyaluronidase were therapeutically applied as a sprading factor in several medical fields for many years (Menzel & Farr, 1998). It was shown that the major soluble hyaluronidase present in bull testes extracts is a fragment of the membrane bound PH-20 enzyme (Meyer, Kreil, & Aschauer, 1997). BTH is an endo-glycanohydrolase (EC 3.2.1.35) degrading HA by cleavage of the β-1,4-glycosidic bond. In addition to HA, BTH also degrades chondroitin, chondroitin-4- and -6-sulphate and, to a small extent, dermatan sulphate. By using ion-spray mass spectrometry, tetrasaccharide and saturated disaccharide fragments were identified as major and as smallest hydrolysis products, respectively (Takagaki et al., 1994; Takagaki, Nakamura, et al., 1994; Saitoh et al., 1995). The mammalian PH-20 was found to be homologous to bee venom hyaluronidase (BVH) showing ca. 30% sequence similarity and conservation of the active site residues (Gmachl & Kreil, 1993; Markovic-Housley et al., 2000).

4.1.3. Bee venom hyaluronidase (BVH)

Bee venom hyaluronidase is one of the major allergens present in bee venom which specifically degrades HA in the extracellular matrix of skin thereby facilitating penetration of venom constituents into the body. Bee venom hyaluronidase (BVH) is a hyaluronate 4-glycanohydrolase (E.C. 3.2.1.35) sharing ca. 30% sequence similarity with the mammalian hyaluronidases. On the basis of sequence similarity mammalian hyaluronidases and BVH are assigned to family 56 of glycosyl hydrolases (Henrissat & Bairoch, 1996). Compared to the human and bovine enzymes BVH lacks a C-terminal domain of ca. 120–150 amino acids (Gmachl & Kreil, 1993). Like BTH, in addition to HA the hyaluronidase from bee venom also degrades chondroitin sulphate. The pH activity profile of BVH reported prevoiusly (Allalouf, Ber, & Ishay, 1975) shows a maximum activity at pH 4.5 and remarkable activity at neutral pH.

Before known crystal structure of a mammalian hyaluronidase, information about the catalytic mechanism and the structure of human hyaluronidases can merely be transferred from the crystal structures of the bee venom hyaluronidase (Markovic-Housley et al., 2000) and the wasp venom hyaluronidase (Skov et al., 2006). In addition fold recognition studies revealed a significantly higher structural similarity than expected from the amino acid sequence enabling the construction of 3D model structures of the human hyaluronidases (Jedrzejas & Stern, 2005) and bovine PH-20, which exhibits a sequence identity of 65% to human PH-20. The overall model structures of the human hyaluronidases resemble each other closely with the typical barrel-shaped catalytic domain exhibiting a broad binding groove for the substrate and a linker region connecting the catalytic domain to the C-terminal domain (Jedrzejas & Stern, 2005). In all hyaluronidases the binding groove is lined by electropositive and hydrophobic residues favoring the binding of substrate molecules with electronegative and hydrophobic patches. Model structures of the C-terminal domains were predicted by an *ab initio* approach due to missing similarity to any of the known 3D structures and are therefore rather unreliable.

Nowadays crystal structure of one human hyaluronidases (Hyal-1) is available (Chao, Muthukumar, & Herzberg, 2007), in which add some information about structure and function and also predication the reason for the different catalytic condition of each hyaluronidase. The crystal structure of Hyal-1 reveals molecular composed of two closed associated domains: the N-terminal catalytic domain and smaller C-terminal domain (Fig. 3) (Chao et al., 2007). The catalytic domain adapts a distorted (β/α)8-barrel fold similar to that of the BVH structure (Markovic-Housley et al., 2000; Chao et al., 2007; Schultz, Copley, Doerks, Ponting, & Bork, 2000; Hulo et al., 2006; Louie, Yang, Bowman, & Choe, 1997; Jedrzejas & Stern, 2005). The catalytic mechanism of the hyaluronidases (glycosyl hydrolase family 56) resembles that of the glycosyl hydrolases belonging to families 18 and 20 (Terwisscha van Scheltinga et al., 1995; Drouillard, Armand, Davies, Vorgias, & Henrissat, 1997), which involves double displacement at C1 next to the β (1→4) glycosidic bond to be cleaved. This involves a single Glu residue within the enzyme, the only catalytic residue, as the proton donor (acid). Also involved is a carbonyl group of the hyaluronan (HA) N-acetyl-D-glucosamine as a unique type of nucleophile. Thus the substrate participates in the mechanism of action of its own catalysis. Simply, first association with cleavage of the glycosidic bond, a glutamic acid residue (Glu131 in Hyal-1) transfers a proton to the C4 oxygen of the leaving HA fragment. Next, an incoming water molecule replaces the leaving HA fragment. The disposition of active site residues in Hyal-1 suggests that Glu131 and Tyr202 polarize the water molecule for the nucleophilic attack on C1, thus completing the hydrolysis (Chao et al., 2007).

Although human hyaluronidases exhibit 33–42% sequence identities and even higher conservation of active site residues, the enzymes differ in their catalytic efficiencies and pH profiles. For example, bovine PH-20 (61% sequence identity to the human counterpart) is ~400-fold more active than Hyal-2 at acidic conditions (Vigdorovich, Miller, & Strong, 2007). Human Hyal-1 hydrolyzes HA in a size-independent manner to tetrasaccharides, whereas hHyal-2 specifically degrades large HA polymers into 20 kDa fragments (Harada & Takahashi, 2007). The Hyal-1 activity optimum is at pH 3.8, Hyal-2 at pH 5.6–7.0 (Frost et al., 1997; Vigdorovich et al., 2007; Harada & Takahashi, 2007), and the bovine PH-20 pH profile is bimodal, with maxima at 4.5 and 7.5 (Frost et al., 1997). With agreement with modeling structure done before (Jedrzejas & Stern, 2005), the sequences of the reaction centers are conserved in all family members, residues at the peripheries of the active site clefts are more divergent. Such variations may be responsible for the different catalytic properties of the human hyaluronidases (Chao et al., 2007; Jedrzejas & Stern, 2005). By comparison, the primary substrates of Hyal-4 were reported to be chondroitin and chondroitin sulfate (Stern & Jedrzejas, 2006), which differ from HA in the anomeric form of the C4 hydroxyl of the N-acetyl-D-glucosamine unit. Human Hyal-4 contains a cysteine residue instead of Tyr247, which is the only departure from the conserved catalytic machinery of the hyaluronidases. This change may contribute to the apparent different substrate specificity of human Hyal-4.

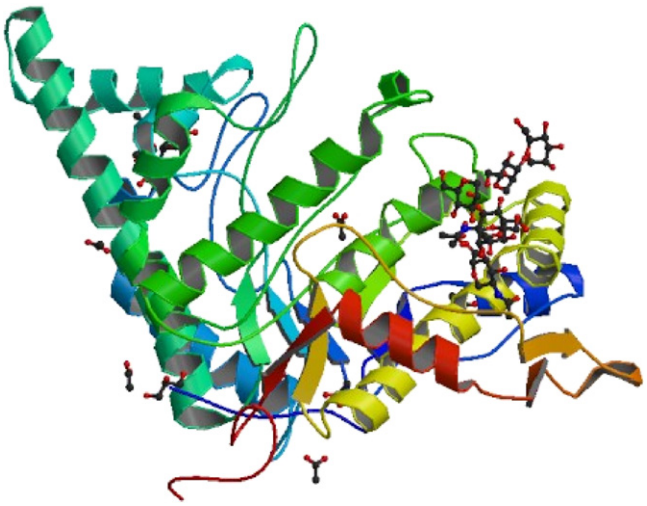

Fig. 3. 3D struture of human hyaluronidase type 1 (Chao et al., 2007).

4.1.4. Other venom hyluronidases

There are another animal venoms have hyluronidase activity (Tan & Ponnudurai, 1992a). The enzymatic properties, including hyluronidase, have been extensively studied by Tan et al. (Tan & Ponnudurai, 1992b). Snake hyluronidase can degrade hyaluronan, chondroitin, chondroitin-4- and -6-sulfate, producing various oligosaccharides, mainly tetrasaccharides. High amounts of hyluronidase are present in scoiopn venom (Tan & Ponnudurai, 1992a). The enzyme is also present in the venom of tarantula and in the venom of the poisonous brown recluse spider (Wright, Elgert, Campbell, & Barrett, 1973; Girish, Jagadeesha, Rajeev, & Kemparaju, 2002). Transglycosalation properties have been observed with hyluronidase from snake venom ("http://glycob.oxfordjournals.org/cgi/content/full/8/7/719" \l "bbr27_1#bbr27_1" Weissman (1955)). Vemom of social wasps was found to contain high levels of hyaluronidases activity, whereas venom from ants contains low levels of activity (Schmidt, Blum, & Overal, 1986). In contrast lizard venom hyluronidase specific degrading hyaluronan while has no activity toward chondroitin-6-sulfate, dermanta sulfate or heparin and only weak activity toward chondroitin-4-sulfate (Tu & Hendon, 1983). A hyluronidase has been purified and characterized from stonefish and it can degrades only hyaluronan producing tetra-, hexa-, octa-, and decasaccharides while no activity on chondroitin sulfate or dermanta sulfate (Poh, Yuen, Chung, & Khoo, 1992; Sugahara et al., 1992).

4.2. Prokarytic (bacterial) hyaluronidases

The hyaluronate lyases from *S. pneumoniae* and *S. agalactiae*, respectively, are supposed to degrade the polymeric substrate via a processive mode of action (Li et al., 2000; Li & Jedrzejas, 2001) (Table 3). In this processive mechanism of action the enzyme probably binds randomly to a HA molecule and cleaves it into two pieces by the aforementioned β-elimination process (initial endolytic cleavage). The resulting unsaturated fragment, containing the reducing end of the primary HA molecule, leaves the catalytic cleft of the enzyme, whereas the other fragment remains in the cleft, where it is translocated by one disaccharide unit toward the reducing end. By this translocation process the truncated HA chain is positioned in the active site of the enzyme, so that an unsaturated disaccharide product is generated by the following catalytic cleavage. The produced β-DiHA is released from the enzyme, and the remaining HA chain is again translocated in the cleft by one disaccharide unit for the next round of catalysis. The degradation mechanism was initially suggested, based on the analysis of the products of HA degradation (Baker & Pritchard, 2000), and could be supported by elucidation of the 3D structures of the enzymes in complex with hyaluronan fragments of varying length (Jedrzejas et al., 2002; Mello, de Groot, Li, & Jedrzejas, 2002). With respect to the direction of hyaluronan degradation the data reported in the literature are contradictory. Analysis of the structural data of the enzyme in complex with substrate suggests that the degradation takes place from the reducing to the nonreducing end of a HA chain, as described above (Jedrzejas et al., 2002). However, based on the analysis of the degradation products (Baker & Pritchard, 2000), the degradation proceeds from the nonreducing to the reducing end of the substrate chain. Therefore this proposed mechanism differs from that suggested by Jedrzejas et al. (2002) in so far as – after the initial endolytic cleavage – not the saturated fragment but the unsaturated fragment remains at the enzyme to be further degraded to disaccharides.

4.3. Invertebrate (Leech) hyaluronidases

This type of hyaluronidases can be extracted from the heads of leeches. The medicinal leech is commonly used as the source of this enzyme, but a tropical Asian species of leech can serve as an alternative source (Budds, Edwards, Olavesen, & Gacesa, 1987). Leech hyaluronidases degrade hyaluronan, producing a tetrasacchraide with gluconic acid at the reducing end. It shows no activity toward chondroitin and chondroitin-4- or -6-sulfate (Linker, Meyer, & Hoffman, 1960). Leech hyaluronidases haven't transglycoslation properties (Welssmann et al., 1955). A noval hyaluronan degrading enzyme has been isolated, purfied, and characterized from Antarctic krill (Karlstam and Ljungloef, 1991) in which that enzyme acts as an endo-B-glucuronidase.

5. Activity assays of hyaluronidases

A variety of assay methods has been devised for the determination of hyaluronidase activity over the years. The methods can be grouped into chemical, physicochemical and biological methods.

5.1. Chemical methods

5.1.1. Quantification of reducing sugars

The determination of the increase in reducing sugars resulting from the cleavage of the glucosidic bonds of hyaluronan molecules has been widely used for the quantification of hyaluronidase activity (Meyer, 1947). The reductimetric procedure provides a method to measure product formation. However, it is more sensitive than the physicochemical methods and requires highly purified substrate (Rapport, Meyer, &Linker, 1950).

5.1.2. Determination of reducing N-acetylglucosamine (NAG)

As the hyaluronidases of the enzyme classes EC 3.2.1.35 (hyaluronate 4-glycanohydrolases) and EC 4.2.2.1 (hyaluronate lyases) liberate N-acetylglucosamine end groups from hyaluronan, these enzymes can be assayed by a colorimetric method based on the Morgan–Elson reaction for the determination of carbohydrates containing terminal Nacetylhexosamine moieties (Morgan & Elson, 1934). This colorimetric method (MorganIntroduction 20 Elson assay), which was optimised by Reissig, Storminger, and Leloir (1955) and further modified for the estimation of hyaluronidase acitivity in human plasma (Gacesa, Savitsky, Dodgson, & Olavesen, 1981; Muckenschnabel, Bernhardt, Spruß, Dietl, & Buschauer, 1998), represents one of the most frequently used hyaluronidase assays. Recently new modifications of this colorimetric assay were published (Asteriou et al., 2001; Takahashi, Ikegami-kawai, Okuda, & Suzuki, 2003). NAG moieties at the reducing ends of HA and its fragments generated by the hydrolase (and transglycosylase) activity of hyaluronidase are determined after derivatisation to a red-colored product with p-dimethylaminobenzaldehyde (Ehrlich's reagent). In contrast to the physicochemical methods, which are based on the detection of residual high-molecular mass substrate and measure the hyaluronidase induced changes in the physicochemical properties of the substrate solution; by the colorimetric method product formation is detected. Therefore, in the Morgan–Elson assay hyaluronidase activity can be quantified according to the definition of the International Union of Biochemistry by defining 1 unit (U) as the amount of enzyme that catalyses the liberation of 1 μmol of NAG at the reducing ends of sugars per min under specified conditions. For bovine testicular hyaluronidase it was shown that, according to the aforementioned definition, 0.1 mU (0.1 nmol NAG/min) is equivalent to approximately 1 TRU and 1 IU, respectively, when using HA from rooster comb as substrate (Oettl, Hoechstetter, Asen, Bernhardt, & Buschauer, 2003; Meyer & Rapport, 1951). However, this relation may not be generalised, since it may depend on various factors like the molecular mass of the substrate or the pH of the incubation medium (Hoechstetter et al., 2001). In addition, it has to be pointed out that an entirely different relation may be observed in case of

bacterial hyaluronidases due to their different mechanism of HA degradation.

In addition to the classical methods quantitative hyaluronidase assays are also performed by spectrophotometry of complexes between hyaluronan and dyes (Benchetrit, Pahuja, Gray, & Edstrom, 1977; Turner & Cowman, 1985; Homer, Denbow, & Beighton, 1993), chromatography (Cramer & Bailey, 1991; Cramer, Bailey, Bailey, & Miller, 1994; Vercruysse, Lauwers, & Demeester, 1994), capillary zone electrophoresis (Pattanaargson & Roboz, 1996) or polyacrylamide gel electrophoresis (Ikegami-Kawai & Takahashi, 2002). Also a variety of assays using fluorogenic hyaluronate as substrate have been reported (Nakamura et al., 1990; Calabro, Benavides, Tammi, Hascall, & Midura, 2000; Krupa, Marie Butler, & Mort, 2003; Nagata et al., 2004). An ELISA-like assay for hyaluronidase and hyaluronidase inhibitors was developed by the group of Robert Stern (Stern & Stern, 1992; Frost & Stern, 1997; Nawy, Csoka, Mio, & Stern, 2001). A new method combining agarose gel electrophoresis and enhanced chemoluminescence-assisted detection was recently described by Müllegger, Reitinger, and Lepperdinger (2001) as a sensitive assay for the Hyal-2 enzyme, which is difficult to assay by most other methods due to its unusual substrate specificity. Furthermore zymographic methods are used for the detection of hyaluronidases (Liefländer & Stegemann, 1968; Steiner & Cruce, 1992; Miura, Yamagata, Miura, Harada, & Yamagata, 1995) and hyaluronidase inhibitors (Mio, Csoka, Nawy, & Stern, 2001).

5.2. Physicochemical methods

5.2.1. Mucin clot prevention method

This method is based on the observation that native hyaluronate in acid solution precipitates with protein in a fibrous clot. After incubation of hyaluronate with hyaluronidase the character of the precipitate changes from fibrous to flocculent, finally no precipitate is obtained (Robertson, Ropes, & Bauer, 1940; McClean, 1943). One unit of hyaluronidase was defined as the amount of enzyme, which prevents the clotting of 0.4 mg of crude hyaluronate (Harris & Harris, 1950).

5.2.2. Spinnability method

The spinnability of dialised bovine synovial fluid is destroyed by hyaluronidases (Chen et al., 2009). The spinnability is determined by a special apparatus which measures the length to which a filament of the substrate solution can be drawn at a standard velocity. One unit of enzyme was defined as the amount of enzyme, which reduced the spinnability to 50% of the initial value in 20 min under standard conditions (Gunter, 1949).

5.2.3. Viscosity reduction method

In this method the reduction in viscosity of a solution of hyaluronic acid, induced by the action of hyaluronidase, is measured. The viscosimetric method (Madinaveitia & Quibell, 1940) has been extensively employed in various modifications (Meyer & Rapport, 1952). Viscosimetric units of hyaluronidase have been established by defining 1 unit as the amount of enzyme required to reduce the viscosity of a HA solution to half the initial viscosity under specified conditions (Madinaveitia & Quibell, 1940; Meyer, 1947). However, although the viscosimetric method proved to be sensitive and accurate, comparison of the results obtained by different research groups turned out to be difficult, since the half viscosity reduction times were found to be highly dependent on the initial viscosity of the hyaluronate solution, which varies depending on the molecular mass of the respective HA preparation (Alburn & Whitley, 1951). In the viscosimetric assay, which is used for the standardisation of hyaluronidase preparations, the activity of hyaluronidase is quantified in terms of International Units (IU) by comparing the rate of viscosity reduction induced by the hyaluronidase preparation to be quantified with the rate obtained with the "International Standard for Hyaluronidase" (Humphrey, 1957) or a reference preparation calibrated in International Units. A viscosimetric approach to determine the activity of hyaluronidase expressed as mol of bonds broken per unit time (Vercruysse, Lauwers, & Demeester, 1995).

5.2.4. Turbidimetric method

The turbidimetric assay relies on the observation (Kass & Seastone, 1944) that hyaluronate of high-molecular mass forms precipitates with diluted acidified serum, whereas depolymerised hyaluronate remains clear under the same conditions. The average molecular mass at which turbidity formation disappears is reported to lie between 6 and 8 kDa (Rapport et al., 1950). Several modifications of the method have been described. In addition to horse serum, rabbit serum, human serum or human plasma also purified protein fractions as horse serum albumin or bovine plasma albumin are reported to serve as precipitating protein reagents (Meyer, 1947; Dorfman & Ott, 1948; Dorfman, Ott, & Whitney, 1948; Tolksdorf, McReady, McCullagh, & Schwenk, 1949; Schmith & Faber, 1950). Other turbidimetric methods are based on the formation of insoluble complexes between high-molecular mass HA and quarternary ammonium salts (Scott, 1955). Hyaluronidase assays using cetyltrimethylammonium bromide or cetylpyridinium chloride as precipitating agents are described (Di Ferrante, 1956), respectively. In the turbidimetric assay hyaluronidase activity is expressed in turbidity reduction units (TRU): 1 TRU is defined as the amount of enzyme which will reduce the turbidity produced by 0.2 mg of HA to that produced by 0.1 mg of HA within 30 min under specified conditions (Kass & Seastone, 1944; Meyer, 1947). However, by some authors, employing the turbidimetric method, slightly modified arbitrary units were defined: (1) to overcome the problem that different preparations of hyaluronate may produce different turbidities at a given concentration, Tolksdorf et al. (1949) introduced a new unit based on a standard initial turbidity instead of the standard HA concentration (0.2 mg) used in the aforementioned definition: 1 unit was defined "as the amount of enzyme, which will hydrolise one half of a quantity of substrate sufficient to cause a turbidity corresponding to $50 \pm 5\%$ light transmission" (Tolksdorf et al., 1949), (2) by Gerlach and Köhler (1972) 1 unit was defined as the amount of enzyme, which produces a 50% reduction of the turbidity given by the initial quantity (0.1 mg) of hyaluronate.

In 1957 an "International Standard for Hyaluronidase" was established: tablets were prepared from lyophilised bovine testicular hyaluronidase blended with lactose, and their activity was assayed turbidimetrically. On the basis of this examination the International Unit (IU) of hyaluronidase was defined as the activity of 0.1 mg of the international standard preparation, which is almost equal to 1 TRU (Humphrey, 1957). The turbidimetric method, using horse serum as precipitating agent, is used for the standardisation of hyaluronidase preparations. Enzyme activity is quantified in terms of "USP Hyaluronidase Units" by comparing the turbidity reduction induced by the hyaluronidase preparation to be quantified with the turbidity reduction obtained with the "USP Hyaluronidase Reference Standard".

5.3. Biological methods

5.3.1. Spreading effect method

Biological assays measuring the effect of hyaluronidase coadministration on the spreading of an indicator dye injected into the skin of animals were described previoualy. Although the spreading assay cannot be used as an accurate quantitative assay of hyaluronidase (Meyer & Rapport, 1952), it is the most direct method for the determination of the therapeutical efficacy of hyaluronidase preparations, which are intended to be applied as spreading factors

to improve the absorption of drugs. In this context it is noteworthy that a spreading assay developed (Jaques, 1953) showed results comparable to those obtained by viscosimetric and turbidimetric in vitro methods (Humphrey & Jaques, 1953). Decapsulation of mucoid strains of streptococci, another biological method is based on the ability of hyaluronidase to degrade the mucoid capsule of group A and group C streptococci (Fulton, Marcus, & Robinson, 1950; Meyer & Rapport, 1952).

6. Biological functions of hyaluronidases

6.1. Tumor development and progression

Hyaluronidase is employed therapeutically since many years. The various detectable effects of hyaluronidase are referred to the action of this enzyme. Hyaluronidase depolymerises the mucopolysaccharide hyaluronic acid, a component of the mucoprotein ground substance or tissue cement. Hyaluronidase thereby increases in tissues the membrane permeability, reduces the viscosity and renders the tissues more readily permeable to injected fluids (spreading effect). The effects of hyaluronidase enables this enzyme to be used therapeutically to increase the speed of absorption and to diminish discomfort due to subcutaneous or intramuscular injection of fluid, to promote resorption of excess fluids and extravasated blood in the tissues and to increase the effectiveness of local anaesthesia. Hyaluronidase is widely used in many fields, i.e. in orthopaedia, surgery, ophthalmology, internal medicine, oncology, dermatology, gynaecology.There is no doubt about the involvement of both hyaluronan and the catabolizing enzymes, the hyaluronidases, in tumor development and progression. However, data about the effects of hyaluronidases on cancer growth, progression, spread and outcome are controversial. Hyal-1 and Hyal-2 can in some cases like in tobacco-related lung cancers and cancers of the upper airways function as tumor suppressor genes (Frost, Csoka, & Stern, 1996; Frost et al., 2000; Chang, 2002; Duterme, Mertens-Strijthagen, Tammi, & Flamion, 2009), but were in other types of tumors described as oncogenes (Novak, Stylli, Kaye, & Lepperdinger, 1999; Lokeshwar et al., 2001; Lokeshwar, Schroder, Carey, Soloway, & Lida, 2002; Lokeshwar, Cerwinka, & Lokeshwar). Increased expression of HYAL1 mRNA and higher levels of Hyal-1 are documented in many diseases, and in bladder and prostate cancers. The enzyme is responsible for the production of angiogenic HA fragments. Because of its presence in urine and change in expression level in bladder and prostate cancers, the use of Hyal-1 as a marker of these diseases has been considered (Posey et al., 2003; Lokeshwar, Cerwinka, & Lokeshwar; Aboughalia, 2006).

Hyal-2 is assumed to be responsible for the strong oncogenic potential of Jaagsiekte sheep retrovirus as it acts as the cell surface receptor for the virus (Rai et al., 2001). This associated with lung cancers, and activates the growth and invasion of brain tumors (Novak et al., 1999; Rai et al., 2001). However at recent work by making inactivating mutations to the catalytic residues of hHyal2, it is found that hyaluronidase activity was dispensable for its receptor function (Vigdorovich et al., 2007). PH-20 was expressed in a series of malignant tumors, e.g. breast, prostate and laryngeal cancers and also in cell lines derived from melanomas, glioblastomas and colon carcinomas (Stern, 2005). Like Hyal-1, also the Hyal-2 protein is involved in tumor formation: it can function as either an oncogene or a tumor suppressor gene product. At the one hand, overexpression of Hyal-2 is reported to accelerate the formation of murine astrocytoma cells (Novak et al., 1999) and on the other hand, Hyal-2 was found to accelerate apoptosis (Chang, 2002). Testicular enzyme is used as a 'spreading factor', to improve better penetration of chemotherapeutic drug into tumors. Drawback of this approach is hyaluronidase developed symptoms of immediate type 1 allergic reaction.

The PH-20 protein plays a major role in mammalian fertilization. The male and female guinea pig immunized with PH-20 showed 100% contraception and the effect was long lasting and reversible (Primakoff, Hyatt, & Myles, 1985; Primakoff, Lathrop, Woolman, Cowan, & Myles, 1988). This suggests PH-20 can be effectively used as a contraceptive vaccine. Commercially available PH-20 preparations from bovine testes have been used for many years for experimental and therapeutic purposes, e.g. in orthopedia, ophthalmology and internal medicine (Spruss, Bernhardt, Schönenberger, & Schiess, 1995; Menzel & Farr, 1998; Muckenschnabel, Bernhardt, Spruß, & Buschauer, 1998; Muckenschnabel et al., 1998). The level of HA surrounding tumor cells often correlates with tumor aggressiveness and overproduction of HA enhances anchorage-independent cell growth. Loss of hyaluronidase activity, permitting accumulation of HA, which might be one of the several steps required by cells during carcinogenesis. In addition, hyaluronidase expression was seen in various metastatic cell lines, which suggest tumor cells use hyaluronidase as "molecular destroyer" to depolymerize hyaluronic acid in surrounding tissues and facilitate tumor invasion. Hyaluronidase on tumor cells may provide a target for anti-neoplastic drugs (Ghatak, Misra, & Toole, 2002).

Abnormalities in the deposition of HA have been found in the stroma surrounding tumors indicating an aberrant HA metabolism. However, the contribution of stromal HA to the tumor mass varies significantly dependent on the kind of tumor. In the course of tumor progression the HA metabolism is also very likely to change dependent on the different stages of initiation, growth and metastasis (Stern, 2005). At the initial stage of tumor formation high-molecular weight HA can provide hydrated pathways for cell migration and diffusion of nutrients. If supply by diffusion becomes insufficient low molecular weight HA fragments produced through the action of Hyal-2 could promote angiogenesis (West, Hampson, Arnold, & Kumar, 1985). This phenomenon of the angiogenic switch (Folkman, 2002) could account for some of the anomalies described in literature. The situation is severely complicated by the fact that HA is under normal conditions turned over quickly and abnormalities in the concentration of HA can reflect aberrations both in the anabolic and in the catabolic pathways. Furthermore, the hyaluronidase activity is controlled in the tissue by biological inhibitors that are hardly characterized (Mio & Stern, 2002).

In addition to the effects mediated by the degradation products of HA, GPI-anchored hyaluronidases (PH-20, Hyal-2 and, presumably Hyal-4) themselves can participate in signal transduction events. As shown for PH-20 the interaction between HA and a hyaluronidase can increase intracellular Ca^{2+} levels resulting in an amplification of the zona-pellucida induced acrosome reaction (Cherr et al., 2001). The interaction of signaling molecules like Src-family tyrosine kinases with GPI-anchored proteins appears to take place in glycosphingolipid-cholesterol microdomains, so called "lipid rafts" (Kasahara & Sania, 2000; Zhang et al., 2009). Within these membrane domains aggregation and cross-linking of GPI-anchored proteins, e.g. upon binding to hyaluronic acid, are supposed to trigger intracellular signaling events (Cherr et al., 2001).

Although rather little is known about the oncogenic potential of PH-20, Hyal-2 was shown to mediate transformation of fibroblasts by virus-induced disturbance of the interaction between Hyal-2 and RON receptor tyrosine kinase (Danilkovitch-Miagkova et al., 2003). Many of the contradictory data accumulated during the last years could be resolved if the differential functions of the hyaluronidase subtypes and the interactive pathways of the HA catabolism were better understood. Recently, Hyal extracted from Norway lobster (*Nephrops norvegicus*) was also successfully com-

mercially used (Krishnapillai, Taylor, Morris, & Quantick, 1999a; Krishnapillai, Taylor, Morris, & Quantick, 1999b). The examples of applications of hyaluronidases in the pharmaceutical and food industry show that dispersion and absorption of other drug and treatment of cancer is facilitated considering them as putative virulent factors of pathogenic organisms in cultures.

Interactions between hyaluronan and cells in cancer progression are reviewed previously (Toole, Wight, & Tammi, 2002; Toole, Biswas, & Gross, 1979; Toole, 2002, 2001). Various tumors are surrounded by a connective tissue matrix (stroma) enriched with hyaluronan. The enzyme hyaluronidase appears to interfere with metastasis, the process by which tumors spread from the primary organ in which they arose to other sites in the body, through three mechanisms. Two of the requirements for cancer cells to metastasize are that they must become motile, and that they must have a space into which to move. Hyaluronan accomplishes both of these tasks. Hyaluronan on the surface of cancer cells hydrates, expands, and opens up spaces around cancer cells, enabling them to invade surrounding tissues. One suggestion is that invasive tumor cells, or products secreted by these cells, induce stimulation of hyaluronan synthesis by stromal cells such as fibroblasts or smooth muscle cells (Knudson & Knudson, 1995). An increase of hyaluronan may provide an embryonic-like environment that facilitates tumor cell migration (Knudson et al., 1993; Knudson, 1996), as an enhanced of cell motility (migration) is necessary for cells to become metastatic. Using animal models, rabbit V2 carcinoma was studied (Toole et al., 1979) demonstrating that the accumulation of hyaluronan is correlated with tumor invasiveness.

Hyaluronan also allows tumor cells to protect themselves against immune assaults by such mechanisms as the secretion of non-specific suppressive or anti-inflammatory factors or the shedding of tumor-specific antigens such as lymphocyte-mediated cytolysis (Menezes, McAvoy, & Kubes, 2009). Removal of hyaluronan present in the extracellular matrix of tumor cells by hyaluronidase renders the latter more accessible to effector T cells. Experiments showed in transplanted mice suffering two tumors (3LL lung carcinoma and the B16F10 melanoma) that tumor proliferation is influenced by hyaluronidase 1 (HYAL1). Both tumors developed more slowly, measured by a slower rate of increase in local tumor size and by a prolonged survival time, in presence of higher levels of circulating hyaluronidase concentrations (De Maeyer & De Maeyer-Guignard, 1992). Hyaluronidase treatment destroys the hyaluronate coat surrounding tumor cells and allows lymphocytes to approach the tumo membrane to enhance the cytotoxic action of immune response (McBride & Bard, 1979) and to be more accessible both for tumor-specific cytotoxic-T-cell generation and activity (De Maeyer & De Maeyer-Guignard, 1992).

The hydrodynamic effect of hyaluronan on the extracellular matrix interacts with cell migration, a role mediated via cell surface by hyaluronan-binding sites or hyaluronan receptors (Fig. 4). Hyaluronidase breaks down HA and eliminates as a direct effect of this mechanism cancer cell spread. The receptor for HA, the lymphocyte homing receptor CD44, functions as an anchor to bind HA to the cancer cell surface. CD44 comes in a variety of forms, a short form found on normal cells, and longer variants that occur on motile cancer cells only (Knudson, 1996). A portion of CD44 protrudes through the surface membrane into the cell cytoplasm, where it interacts with the cytoskleleton, the machinery for cell movement. Interactions between hyaluronan and CD44 have been reported to play the critical role in the process of tumor metastasis. They promote the formation of tumor embolisms, which in turn, increase the chances that the tumor cells would be trapped in the lungs (Zhang, Underhill, & Chen, 1995). As shown experimentally, inserting this longer cancer form of CD44 into tumor cells changes them into cancer cells that aggressively metastasize. Hyaluronidase eliminates

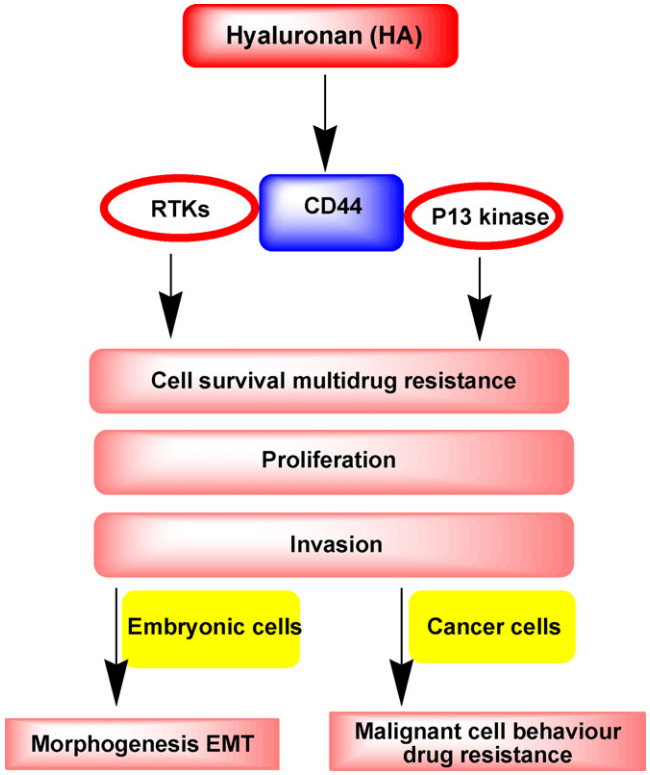

Fig. 4. Effects of hyaluronan (HA) on oncogenic signaling. Endogenous HA–CD44 interaction is required for the formation of constitutive signaling complexes containing lymphocyte homing receptor CD44, activated receptor tyrosine kinases (RTKs), phosphatidylinositol–3-kinase (PI3 kinase), and several other signaling molecules involved in cell survival, proliferation, and invasiveness (Toole, 2001, 2002, 2004).

the cancer cells' ability to move, by converting the long cancer form of CD44 to the short "normal" immobile form. A molecular and cell biology study to determine precisely how hyaluronidase regulates this alternative splicing of CD44 is done in the California Breast cancer research project. Understanding of these events in the regulation of CD44 will finally make it possible to develop a new generation of anticancer drugs to prevent metastases associated with breast cancer progression.

The enzyme causes established breast cancers to shrink. Inoculation of hyaluronidase in mice suffering human breast cancer shrinks the tumor to half its size in four days. The malignant cells cannot maintain motility and thus invasiveness and the tumor cannot progress or grow and must completely apparently undergo regression. These biological mechanisms are still not understood, but may be a consequence of the two functions as mentioned before. Hyaluronidase may thus prevent progression of human breast cancer, and it seems not to have the devastating toxic side effects of other anticancer agents.

Another role of hyaluronidase in the context of cancer may be the inhibition of tumor cell growth by increased tumor necrosis factor (TNF) cytotoxicity. Hyal counteracts transforming growth factor beta mediated (TGF-beta-mediated) TNF-resistance and suppresses TGF-beta1 gene expression. Hyaluronidase antagonizes TGF-beta-mediated inhibition of epithelial cell growth. Both TGF-beta and hyaluronidase are essential for the progression and invasiveness of breast, prostate and other cancers. A stage-dependent expression, as well as a balanced production of these proteins is essential for cancer development and self-protection against TNF cytotoxicity (Chang, 1998; Etesse, Beaudroit, Deleuze, Nouvellon, & Ripart, 2009). It has been shown that hyaluronidase is able to disrupt intercellular adhesion and to chemosensitize tumor

cells by a mechanism, independent of increased drug penetration in cancer chemotherapy.

6.2. Fertilization

The process of fertilization begins when the sperm contacts the outermost egg investment (cumulus cells) and ends with fusion of two haploid pronuclei in the egg cytoplasm (Yanagimachi, 1994; Talbort, Geiske, & Knoll, 1999). This process requires sperm to enter oocyte-cumulus complex (OCC) and pass through the cumulus matrix, sperm hyaluronidase is thought to enable acrosome intact sperm to reach the ZP by hyaluronan hydrolysis (Florman and Ducibella, 2006; Kim et al., 2008). Sperm hyaluronidase SPAM1 (sperm adhesion molecule), PH-20 aids in penetrating the layer of cumulus cells by digesting hyaluronic acid. It was found that PH-20 is expressed in testis in two forms. During fertilization, one form is present on the surface of the acrosomal head of sperm which is tethered to the plasma membrane by the lipid anchor glycosylphosphatidylinorcitol (GPI-anchored). This enables the sperm to penetrate the hyaluronan-rich extracellular matrix of the cumulus cells. The second form is expressed and located in the acrosomal vesicle, a soluble form, which clears the hyaluronan in the ZP rapidly and thereby helps the sperm to reach the oocyte membrane. Sperm hyaluronidase activity relates significantly to the fertilization rate. The ability of sperm to penetrate a highly viscous solution of sodium hyaluronan is correlated with the motality and fertilization deficiency. Hyaluronan penetration is used as a method to prepare and evaluate the functional competence of human sperm in assisted fertilization programs. Two hyaluronidases genes, HYALP1 and SPAM1, are both expressed exclusively in testicular tissues; HYAL3 is highly expressed in testis but also in bone marrow (Csoka et al., 1999). These two tissues retain a stem cell-like state for life in the animal but whether or not HYAL3 participates in the process of fertilization is still not known and needs to be elucidated. A possible participation of HYAL5 in fertilization was noted (Baba et al., 2002; Kim et al., 2005, 2008). However resent work contrary to this previous expectation, HYAL5 is not essential for the fertilization process, including sperm penetration of the cumulus matrix (Kimura et al., 2009). Comparative analysis among wild-type, HYAL5-deficient, and SPAM1-deficient epididymal sperm demonstrate that SPAM1, rather than HYAL5, plays an important role in sperm penetration of the cumulus matrix to reach the ZP surface, although these two hyaluronidases are capable of dispersing cumulus cells from the OCCs. Notably, SPAM1 is likely involved in the entry of sperm into the OCC (Kimura et al., 2009).

7. Conclusions

Hyaluronidases are broadly distributed and neglected enzymes with varying substrate specificities, a wide range of pH optima, different catalytic mechanisms and variety functionalities. Much intensive work have been done on this field, however, there is a lot of work to be done on the enzymology, especially 3D structures and site-directed mutagenesis combined with rigorous kinetics to enhance understanding of binding sites, substrate recognition, and catalytic mechanisms. Cloning of more enzymes would allow classes and relationships between these hyaluronidases to be identified. Further work on the structure of its substrate is required, helped by the existence of highly purified hyaluronidases, to improve understanding of the synergistic interactions between enzymes. There are many unanswered questions concerning regulation of expression, including full gene sequences, extent of coordinate regulation, and molecular mechanisms in response to putative inducers. In summary, future work on hyaluronidases should include: (1) elucidating the structural characteristics that determine specificity (structure–function relationships); (2) isolation, characterization and cloning of insect, and plant hyaluronidases; (3) extending the use of existing and novel hyaluronidases as probes for tumor cell structures; and (4) production of tailor-made hyaluronidases with novel biofunctionalities.

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