Mouse testicular hyaluronidase-like proteins SPAM1 and HYAL5 but not HYALP1 degrade hyaluronan

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Besides SPAM1 (sperm adhesion molecule 1; formerly named PH-20), further hyaluronidase-like proteins, HYAL5 (hyaluronoglucosaminidase 5) and HYALP1 (hyaluronoglucosaminidase pseudogene 1) are also expressed in murine testicular tissue. As they share a high degree of sequence similarity with known hyaluronidases, all three polypeptides could potentially exhibit hyaluronidase activity, a function that is beneficial for spermatozoa in order to penetrate the hyaluronan-rich cumulus, which surrounds the oocyte. Recently, it was reported that SPAM1-deficient mice are fertile and spermatozoa derived from mutant mice still exhibit hyaluronidase activity [Baba, Kashiwabara, Honda, Yamagata, Wu, Ikawa, Okabe and Baba (2002) J. Biol. Chem. 277, 30310–30314]. We have now recombinantly ex-

pressed mouse SPAM1, HYAL5 and HYALP1 in *Xenopus laevis* oocytes and determined their respective expression pattern in testis. Transcripts of all three genes are expressed in seminiferous tubules in regions where maturing spermatogenic cells reside. SPAM1 and HYAL5 but not HYALP1 proteins exhibit hyaluronidase activity at neutral pH. The two active hyaluronidases are both bound to the cell surface via a glycosylphosphatidylinositol anchor. Furthermore, structural characteristics are discussed that are necessary for hyaluronidases in order to exhibit hyaluronan cleavage.

Key words: extracellular matrix, fertilization, glycosaminoglycan, hyaluronan, hyaluronidase, sperm adhesion molecule 1 (SPAM1).

INTRODUCTION

Hyaluronan, a polymer consisting of repeating β -1,4-linked disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid, is a common GAG (glycosaminoglycan) present in the ECM (extracellular matrix) of connective tissues [1]. Hyaluronan is implicated in many physiological processes, including cell migration, proliferation and differentiation. Hyaluronidases, enzymes that hydrolyse hyaluronan to oligosaccharides, have been identified in mammalian, insect and bacterial species [2,3].

Hyaluronan accumulates between cumulus cells [4] together with inter- α trypsin inhibitor [5] as well as a variety of other matrix proteins [6–8]. The jelly-like shell protects the oocyte and facilitates its extrusion at ovulation [9]. Furthermore, the swollen cumulus is more easily captured by the fimbria of the oviduct and thus supports oocyte transportation to the site of fertilization [10]. The tight matrix however is difficult to perforate by mechanical means. Spermatozoa hyaluronidase activity positively correlates with fertilization rate, because spermatozoa that possess high motility and fertilization efficiency penetrate highly viscous solutions of hyaluronan at a higher rate [11,12]. Therefore the motility and spermatozoa hyaluronidase activity are thought to facilitate path finding through the ECM of cumulus cells, since no proteases have yet been implicated in this process [13]. A widely conserved mammalian spermatozoa surface protein, PH-20 [14], also called SPAM1 (sperm adhesion molecule 1) [15,16], is well known for its hyaluronidase activity [17,18]. This GPI (glycosylphosphatidylinositol)-anchored spermatozoa hyaluronidase has multiple roles in fertilization [19]. SPAM1 and sHYAL2 (soluble form of hyaluronoglucosaminidase 2) exhibit activity at both neutral and acidic pH [20], whereas for other

hyaluronidases, enzymatic activity appears to be restricted to either acidic pH, e.g. HYAL1 [21], or neutral pH as known for *Xenopus* XKH1 [22].

Unexpectedly, mice carrying a null mutation in the *Spam1* gene are fertile [23]. *In vitro* fertilization assays showed that mouse spermatozoa, lacking SPAM1, possess a reduced ability to disperse cumulus cells from the cumulus mass, resulting in delayed fertilization solely at the early stages after insemination. In this particular context, spermatozoa extracts derived from mutant mice contain at least one further hyaluronidase, HYAL5, which is active in solutions of both neutral pH and extracellular ionic composition [24].

In the mouse genome, there are seven hyaluronidase-like sequences, six of them clustered in groups of three at two chromosomal sites, at chromosome 9 F1–F2 (*Hyal1*, *Hyal2* and *Hyal3*) and at chromosome 6 A2 (*Hyal4*, *Spam1/PH-20* and *Hyalp1*) [23,25,26]. The orthologous human genes are localized on syntenic regions, on chromosomes 3p21.3 and 7q31.3. *HYALP1* is an expressed pseudogene in humans [27]. In mouse, *Hyalp1* shows more characteristics of a gene that codes for a transcript that can be translated into a polypeptide chain similar to Spam1. The seventh mouse hyaluronidase-like gene, tentatively called *Hyal5*, is localized in close proximity to the cluster at chromosomal region 6 A2. So far, no further hyaluronidase-like gene has been discovered in the human genome at this position. Both genes, *Hyalp1* and *Hyal5*, encode open reading frames for a polypeptide highly similar to the biochemically characterized hyaluronidases [26]. Moreover, these three genes are strongly expressed in testis. So far, none of these murine gene products has been recombinantly expressed and assayed for enzymatic activity. Hence, HYAL5, HYALP1 or both could actually

Abbreviations used: ECM, extracellular matrix; GAG, glycosaminoglycan; GPI, glycosylphosphatidylinositol; HYAL, hyaluronoglucosaminidase; HYALP1, hyaluronoglucosaminidase pseudogene 1; PLC, phospholipase C; RT, reverse transcriptase; SPAM1, sperm adhesion molecule 1.

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be functionally involved in fertilization in the mouse. We therefore expressed these proteins in frog oocytes and studied their enzymatic properties with respect to hyaluronan degradation.

MATERIALS AND METHODS

Frog surgery, oocytes preparation, cDNA construction and cRNA injection

A small lobe of ovary was surgically removed from an anaesthetized adult *Xenopus laevis* female. The ovarian tissue was rinsed in O-R2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄ and 5 mM Hepes, pH 7.8) and individual oocytes were defolliculated manually. The coding regions of *Spaml*, *Hyal5* and *Hyalp1* were cloned into pT7TS (a gift from P. A. Krieg, University of Arizona, Tucson, AZ, U.S.A.), suitable for generating *in vitro*-transcribed mRNA. *Hyal5* cDNA (clone ID IMAGp998B198849.1) was purchased from the German Resource Centre Berlin (RZPD). The protein-coding regions of *Spaml* and *Hyalp1* were amplified by PCR using cDNA derived from mouse testis as a template. To amplify *Spaml*, the following primers were used, 5'-GGTGATCAATGGGAGAGTTGAG-ATTTAAGCACCTC-3' and 5'-CCGCTAGCCTAAGGAGTACT-GACTAGTGTCTTCCG-3'; and to amplify *Hyalp1* the following primers were used, 5'-TCGAGATATCATGTTTATCCAGTGGG-TGACACAG-3' and 5'-GGACTAGTTTAGAAAATGCTTGA-AATCAAAGA-3'.

Approx. 50 ng of capped cRNA was injected into oocytes, using a Nanoliter injector (World Precision Instruments), that were then cultured in O-R2 containing 100 units/ml penicillin and 100 µg/ml streptomycin at 16°C for up to 2 days. GPI-linked proteins were removed from the plasma membranes using 50 milliunits of *Bacillus cereus* PLC (phospholipase C; Molecular Probes) in 30 µl of O-R2 buffer (pH 7.0) containing 4 mM CaCl₂ and 50 µg/ml BSA per oocyte at ambient temperature for 1 h.

Expression analysis

For expression analysis, RNA was extracted as described previously [28]. Mice were dissected. Specimens were carefully rinsed in PBS and snap frozen in liquid nitrogen. Cells or pieces of tissue were homogenized in 4.2 M guanidinium thiocyanate and 100 mM Hepes (pH 7.5). The resulting solution was extracted with phenol/chloroform/methylbutanol (25:50:1) and the aqueous phase was supplemented with 10% (v/v) 2 M sodium acetate (pH 4.4) and 20% (v/v) water and extracted with phenol/chloroform (3:1; v/v). The RNA within the aqueous phase was precipitated by addition of an equal volume of propan-2-ol. The RNA pellet was rinsed with 80% (v/v) ethanol, dried and dissolved in 6 M urea and 100 mM Hepes (pH 7.5). As a final purification step the RNA was precipitated by 50% (v/v) 9 M LiCl. Total RNA (5 µg) derived from various tissues and organs was separated by 1.2% agarose gel electrophoresis and subsequently transferred to Zetaprobe nylon membrane (Bio-Rad). After UV-cross-linking, blots were hybridized with ³²P-labelled cDNA probes. RT (reverse transcriptase)-PCR assays were carried out using specific primer pairs for *β-actin*, *Hyal5* and *Hyalp1* on template cDNAs derived from a range of C57BL/6J mouse tissues, and from a murine Sertoli cell line, TM4 [29]. PCR was performed in 25 µl reaction volumes, containing 1 µM primer and 2.5 units of Pfu DNA polymerase in reaction buffer with MgSO₄ (as recommended by the manufacturer; Fermentas) for 30 and 40 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1 min. Subsequently, the products were analysed on a 2% agarose gel. Quantitative RT-PCR assays were performed using a LightCycler instrument and LightCycler 3.5 software

(Roche). The reactions (15 µl) were carried out using the LC-FastStart DNA Master SYBR Green I kit (Roche), 2 µM primer and 3 mM MgCl₂. After the activation of the enzyme at 95°C for 7 min, 45 amplification cycles were performed at 95°C for 15 s, 58°C for 8 s and 72°C for 12 s. The following oligonucleotide primers were used in order to amplify cytoplasmic *β-actin*: forward 5'-GGCTGTATTCCCCTCCATCG-3', reverse 5'-CC-AGTTGGTAACAATGCCATGT-3'; *Hyal5*: forward 5'-AAAGA-CCCTCGAAATCCAC-3', reverse 5'-CCACATACCATTCCAG-AGG-3'; and *Hyalp1*: forward 5'-TGTCACCTTTGTGGAGAT-CGGA-3', reverse 5'-GCCAACGTGAGAGTAACATTCA-3'. These primer pairs yielded amplification products of 154 bp for *β-actin*, 144 bp for *Hyal5* and 222 bp for *Hyalp1*. The relative abundance of *Hyal5* and *Hyalp1* transcripts was calculated relative to *β-actin*, and the values were normalized to the expression level in testis.

In situ hybridization was performed as described previously [30]. Briefly, testicular tissues from C57BL/6J mice were fixed in PBS containing 4% (v/v) paraformaldehyde. The tissue was embedded in Paraplast Plus (Sigma), sectioned to a thickness of 7 µm and mounted on poly(L-lysine)-coated microscope slides. After treatment with proteinase K and prehybridization, sections were incubated at 50°C with digoxigenin-labelled RNA probes in hybridization buffer containing 50% (v/v) formamide, 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate), 1 × Denhardt's solution (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone and 0.02% BSA), 5 mM EDTA, 50 µg/ml yeast RNA, 0.2% Tween 20, 0.5% CHAPS and 10 µg/ml heparin. Thereafter, sections were washed with hybridization buffer and the probes were detected using anti-digoxigenin alkaline phosphatase Fab conjugate and BM Purple as a substrate (Roche).

Hyaluronidase activity measurement

Enzyme activity was measured as described previously [31]. Briefly, enzymes were mixed with 5-aminofluorescein-labelled hyaluronan and incubated at different pH values (100 mM sodium phosphate buffer containing 50 µg/ml BSA and 0.1% Triton X-100 first adjusted with citric acid to pH 3.5 and then titrated to the appropriate pH with sodium hydroxide). The reaction mixture was separated by 1% agarose gel electrophoresis. The gel was subsequently blotted on to a positively charged nylon membrane (Biodyne PLUS, 0.45 µm; Pall Life Sciences) and the hapten was detected after incubation with anti-fluorescein alkaline phosphatase Fab conjugate (Roche) by means of enhanced chemiluminescence technology (Amersham Biosciences).

Molecular modelling

Tertiary structural models for the hyaluronidase domains of SPAM1, HYAL5 and HYALP1 were generated applying homology modelling of SWISS-MODEL [32] using its alignment interface together with mouse hyaluronidase-like protein sequences of SPAM1 (accession number AAP49832), HYAL5 (accession number BAC55071) and HYALP1 (accession number BAB30316) pre-aligned with the aid of ClustalW and the X-ray structure of bee hyaluronidase (PDB accession code: 1fcv). Images were generated with the aid of DeepView/Swiss-PdbViewer version 3.7 [33].

RESULTS

Structural features

SPAM1, HYAL5 and HYALP1 were found to share conserved elements with hyaluronidase from honeybee. Besides the first

Figure 1 Expression analysis

Hyal5 and *Hyalp1* mRNA expression was determined in various tissues using Northern-blot analyses (**A**), RT-PCR (**B**) and quantitative PCR analysis (**C**). As a loading control, an image of the ethidium bromide-stained agarose gel before transfer to the nylon membrane for hybridization with α - 32 P-labelled probe is shown in the lower part of (**A**). For relative quantification after PCR (**B** and **C**), template cDNAs were diluted with regard to β -actin mRNA content. Amplification was for either 30 or 40 cycles, as indicated. After evaluation of quantitative PCR results, the transcript levels were further normalized to the respective mRNA content of *Hyal5* or *Hyalp1* in testis (**C**).

highly conserved N-terminal domain, all mammalian hyaluronidase-like proteins have a second domain at the C-terminal part of the amino acid sequence. For SPAM1-type hyaluronidases, processing and GPI anchor linkage are needed at the C-terminus in order to bind the enzyme to the outer surface of the plasma membrane [34]. Bioinformatic prediction of whether one of the three different mouse proteins would contain a consensus pattern for GPI linkage yielded inconclusive results [35,36]. Therefore it did not become clear from these theoretical considerations which one of the three hyaluronidase-like genes would potentially code for a hyaluronan-degrading enzyme, and thus is functionally involved in fertilization in mouse.

Expression pattern

Using Northern blots, *Hyal5* as well as *Hyalp1* were found to be expressed in mouse testis (Figure 1A). Like *Spam1*, transcripts of the other two genes are barely detectable within other tissues (Figure 1B). Applying quantitative RT-PCR techniques, trace amounts of both *Hyal5* and *Hyalp1* could be detected in epididymis. This may well be due to spermatozoa present in the highly convoluted epididymal duct. In the TM4 Sertoli cell line [29,37], only *Hyal5* transcripts could be detected with the absence of *Hyalp1* transcript. A small number of *Hyalp1* transcripts as compared with the amount found in testis was also detected in liver, bone marrow, brain, fat, skeletal muscle, lung and spleen as well as in epididymis (Figures 1B and 1C). As shown previously, SPAM1 and HYAL5 proteins appear to be present on acrosome intact spermatozoa [23]. Furthermore, HYALP1 has been found not only in testis and caudal spermatozoa but also in spermatozoa-free epididymis [24].

The precise distribution of *Spam1*, *Hyal5* and *Hyalp1* transcripts in testicular tissue was determined by *in situ* hybridization

(Figure 2). All three hyaluronidase-like transcripts show an equivalent expression pattern of a ring-shaped area within seminiferous tubules (Figures 2B, 2C and 2D). Some of the sectioned tubules show weak to no signals for *Spam1*, *Hyal5* and *Hyalp1* expression. For controls, hybridizations with sense probes were performed in parallel. Low background and no specific signals were observed (Figures 2B, 2C and 2D; inlaid micrographs). In sub-human species, a particular cellular association consisting of spermatogenic cells of a distinct stage occupies a relatively long segment along the length of the tubule. High expression of *Hyal5* or *Hyalp1* transcripts in Sertoli cells appears unlikely since only minor amounts were detected in cultured TM4 cells. We therefore deduced from this expression pattern that transcripts of mouse hyaluronidase-like genes are accumulating in spermatogenic cells, most probably in pachytene and/or leptotene spermatocytes (stage I, II, V and/or VI). No expression was found near or at the basal lamina, where type A and B spermatogonia, preleptotene spermatocytes of stage III and IV reside. Due to the expression pattern, we conclude that all three polypeptides, SPAM1, HYAL5 and/or HYALP1 protein, are present in spermatids and could thus also bind to mature spermatozoa.

Enzymatic activity

Since mouse HYAL5, HYALP1 and SPAM1 have not been recombinantly expressed to examine their hyaluronidase activities, we translated *in vitro*-transcribed cRNA of all three candidates in intact *X. laevis* oocytes. Cell lysates were tested for hyaluronidase activity using a sensitive assay [31]. For HYAL5 and SPAM1, hyaluronidase activity was found in a broad pH range under both acid and neutral conditions. Residual activity could still be measured at pH 7.5 (Figure 3). In contrast with other hyaluronidases such as HYAL2, which primarily generates intermediate length

Figure 2 Transcript localization in testicular tissue

(A) Histological section of murine testis stained with haematoxylin/eosin. Indicated is connective tissue between testicular tubules (ct), the lumen of a tubule (l), the cellular layer containing maturing spermatogenic cells (s) and the lamina propria (lp), the outer layer of tubules. (B–D) mRNA distribution of *Hyal5*, *HyalP1* and *Spam1* in testicular tissue was determined by *in situ* hybridization on histological sections either with antisense or sense probes (inlaid micrographs). Digoxigenin-labelled antisense RNA probes were detected with the aid of anti-digoxigenin Fab alkaline phosphatase complex and BM Purple (Roche) as substrate. Scale bars = 100 µm.

reaction products of approx. 20 kDa in size, both HYAL5 and SPAM1 were found to degrade hyaluronan to short fragments. No hydrolysis was detectable after reaction of HYAL5- and SPAM1-expressing oocytes with GAGs such as chondroitin sulfate A, B and C or heparan sulfate as substrates (results not shown). For recombinant HYALP1, no hydrolysing activity with respect to hyaluronan was detectable although *Hyalp1* and *Hyal5* cRNA were equally well translated *in vitro* in the presence of rabbit reticulocyte lysate (Figure 4). When cell-free expression systems together with microsomal preparations were used for the translation of cRNAs that encode well-characterized hyaluronidases, in no case were enzymatically active protein products obtained. Hence, we extensively tested HYALP1-expressing oocytes or corresponding culture supernatants, in order to detect the presence of trace amounts of hyaluronan-degrading activity. Besides

Figure 4 *In vitro* translation of *Hyal5* and *HyalP1* cRNAs

After protein synthesis with the aid of a cell-free reticulocyte system (Novagen), 35 S-labelled protein products were separated by SDS/PAGE and detected by means of fluorography and phosphorimaging.

Figure 5 Subcellular localization of HYAL5, HYALP1 and SPAM1

Hyal5, *HyalP1* and *Spam1* cRNAs respectively were translated in intact *Xenopus* oocytes. Oocytes were incubated for 2 days in O-R2. Subsequently, healthy oocytes were subjected to PLC treatment (indicated by +) or phospholipase buffer without enzyme (–). The supernatant of these reactions was mixed with fluorescein-labelled hyaluronan and soluble hyaluronidase activity was determined using standard conditions at pH 6.5.

SPAM1 and HYAL5, we observed that the positive controls, human PH-20, HYAL1, honeybee hyaluronidase, *Xenopus* XKH1 [22] and XHyal2 [38], were enzymatically active when expressed in *Xenopus* oocytes. Yet no hyaluronan-hydrolysing activity was detectable after *Hyalp1* cRNA injection either in cells or in the culture supernatant. Furthermore, chondroitin sulfate A and C as well as heparan sulfate were not depolymerized by HYALP1-expressing oocytes (results not shown).

Next, frog oocytes expressing HYALP1, HYAL5 and SPAM1 were incubated with PLC in order to test whether the enzymes had been linked via GPI anchors to the cell surface. In the cases of HYAL5 and SPAM1, enzyme activity was found in the supernatant of PLC-treated oocytes (Figure 5). HYAL2, a hyaluronidase that is normally bound to the plasma membrane via a GPI anchor

Figure 3 Hyaluronidase activity of HYAL5- and HYALP1-expressing *X. laevis* oocytes

Oocytes injected with cRNA encoding *Spam1*, *Hyal5* or *Hyalp1* and uninjected controls (oocyte) were cultured in 200 µl of O-R2 buffer for 24 h. Homogenized oocytes expressing hyaluronidase-like proteins were incubated with 20 ng of 5-aminofluorescein-labelled hyaluronan for either 16 or 49 h respectively. Hyaluronidase activity was tested at different pH values as indicated.

Figure 6 Structural models

(A) Mouse hyaluronidase-like protein sequences, SPAM1 (accession number AAP49832), HYAL5 (accession number BAC55071) and HYALP1 (accession number BAB30316), were pre-aligned using ClustalW and the resulting alignment was finally adjusted manually. Amino acid residues identical in all three sequences were box-shaded. (B) On the basis of the X-ray structure of bee hyaluronidase (PDB accession 1FCV), three-dimensional structural models for SPAM1, HYAL5 and HYALP1 were generated (upper row). Residues that were marked with ○, ●, ■ and ▲ in (A) were found to be located opposite and in close proximity to the active centre (ac) within the tapering substrate-binding cleft. These appear to interact directly with the substrate. The three-dimensional models were slabbed along the line drawn in the frontal view of the bee hyaluronidase (bee hyase) structure. Surfaces were drawn in light grey; interior parts of the molecules in dark colour. Side projections of the active centre reveal variant geometries of the active centres of active hyaluronidases (bee hyaluronidase, HYAL5 and SPAM1) and inactive HYALP1.

and inactive at neutral pH [39], was however found to exhibit weak enzymatic activity also at neutral pH, when recombinantly expressed in its soluble form, sHYAL2 [20]. In the case of HYALP1-expressing oocytes, no hyaluronan-degrading activity was detectable in the supernatant. Notably, the rat homologue of SPAM1, 2B1 glycoprotein (PH-20), is also not rendered free from the cell surface by PLC treatment [40]. We therefore have to admit that HYALP1 could be linked to the membrane and only exerts enzymatic activity after being released by a yet unknown process. In conclusion, HYAL5 and SPAM1, after entering the secretory pathway, are sorted and eventually bind through a GPI anchor to the plasma membrane as active hyaluronidases.

DISCUSSION

In testicular extracts, hyaluronidase activity has been recognized previously by biochemical means [41]. Molecular cloning of the hyaluronidase present in bee venom [17] revealed a strong similarity with a known protein that is localized on the posterior head of human spermatozoa, first characterized by means of a monoclonal antibody, and was therefore called PH-20 [14]. PH-20 from humans exhibits strong and robust hyaluronidase activity at neutral pH [18,42,43]. As recently described, point mutations not only in *Spam1*, but also in other hyaluronidase-like genes such as *Hyal5* and *Hyalp1*, correlate with spermatozoa dysfunction

[26]. As pointed out above, no further gene that potentially would encode hyaluronidase-like proteins other than HYAL1–4 and PH-20/SPAM1 has been discovered in the human genome to date. Besides mouse, *Hyal5* is also present in rat (accession number BC091219). This suggests that further gene duplication has occurred in rodents [26]. Yet, mature spermatozoa of SPAM1-deficient mice still exhibit hyaluronidase activity. It could be used to fertilize eggs [23] and is able to disperse cumulus cells from the cumulus mass [24]. In light of our results, we assume that this is due to functionally active HYAL5 present on the surface of *Spam1*^{−/−} spermatozoa. We furthermore conclude, after having shown that SPAM1 and HYAL5 are both functionally active hyaluronidases that both enzymes participate in spermatozoa fertilization of the egg.

The biological role for HYALP1 remains to be defined. SPAM1, HYAL5 and HYALP1 (39–64 % identical; 64–81 % similar) also share identical residues and a high degree of similarity with other well-characterized hyaluronidases such as human PH-20 or bee hyaluronidase. The most remarkable structural feature of hyaluronidase is a large groove [44] (Figure 6). The amino acid residues of the groove that were found to come in close proximity to the substrate are arranged in well-conserved sequence blocks, and we thus consider them functionally relevant for proper positioning of the sugar polymer. In particular, we propose that four more amino acid moieties (indicated by the symbols shown in Figure 6A) that are located in close proximity to the active centre

control the positioning of the substrate at the active centre. Two amino acid residues were found conserved in all three proteins (open and closed circles in Figure 6A). The other two are different in HYALP (labelled with a square and a triangle in Figure 6A). Molecular modelling revealed a close relationship between the geometries of the active centres of bee hyaluronidase, SPAM1 and HYAL5. However, major spatial differences were evident in the structure of HYALP1, in particular amino acid moieties that appear to be important for the proper positioning of the substrate with regard to the acidic amino acid moieties within the active centre (Figure 6B). The active sites of these enzymes consist of aspartate and glutamate residues interspaced by one amino acid residue [45,46]. Notably, HYAL5 and SPAM1, yet also HYALP1, show this particular feature. Since both active centre residues are present in HYALP1, we assume that the protein, although hydrolysis of hyaluronan was undetectable, might exhibit some yet unknown enzymatic activities. For the substrate binding groove of HYALP1 that showed a high resemblance to the structures of bee hyaluronidase and the structural models of other known hyaluronidases, binding of hyaluronan or other GAGs could indeed be possible. Human PH-20 exhibits many more functions besides hyaluronan degradation [19]. Whether HYALP1 is functionally involved in metabolism of GAG or plays some role during spermatozoa maturation will be investigated in future experiments.

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