Alpha-2 macroglobulin as a region-specific secretory protein in male reproductive tract and its dynamics during sperm transit towards female spermatheca in the blue crab

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Abbreviations: AC, acrosome; AG, androgenic gland; DF, decapacitation factor; DSD, distal spermatic duct; ED, ejaculatory duct; MSD, middle spermatic duct; PSD, proximal spermatic duct; SD, spermatic duct; α2M, alpha-2 macroglobulin

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Abstract

We found a 250-kDa protein that existed in middle spermatic duct (MSD) fluid of *Portunus pelagicus* in a region-specific manner. The N-terminal and partial amino acid sequences revealed that this MSD-specific protein showed a high similarity with a plasma-rich protein, alpha-2 macroglobulin (α2M), termed Ppα2M. Ppα2M was a large glycoprotein enriched in mannose and *N*-acetylglucosamine. The expression of *Ppα2m* mRNA was detected in spermatic duct (SD), androgenic gland (AG) and hematopoietic tissue, while the protein expression was rather specific to the apical cytoplasm of MSD epithelium. Interestingly, Ppα2M was also found within the matrix of the acrosome (AC) of MSD sperm. Distally, Ppα2M was removed from spermathecal sperm membrane, while its level was kept constant in the sperm AC. These results suggest that, after translated, glycosylated in the epithelium of spermatic ducts, and secreted into MSD fluid, Ppα2M is taken up by the MSD sperm, and removed from fertilizable sperm in the spermatheca in the course of ductal transit of sperm toward female spermatheca.

We also demonstrated that Ppα2M showed a protease inhibitor activity. Since accumulated within AC, Ppα2M may thus serve an inhibitory function for inducing premature acrosome reaction that partly happens during sperm maturation process in this curb species.

Abbreviations: α2M, alpha-2 macroglobulin; PSD, proximal spermatic duct; MSD, middle spermatic duct; DSD, distal spermatic duct; ED, ejaculatory duct; SD, spermatic duct; AG, androgenic gland; AC, acrosome

Introduction

In mammals, secretory proteins from epididymal epithelium are acquired onto the sperm membrane during sperm maturation (Frenette et al, 2001; Carmona el al, 2002; Chen et al, 2006). Some epididymal proteins are proposed to function as sperm binding ligands, and others as preventing factors of the early sperm activation, namely decapacitation factors (DFs), which are removed after sperm enter female tract (Ni et al, 2009). Human seminal plasma DFs not only mask sperm binding ligand, but also inhibit tyrosine phosphorylation in sperm, which is a universal biomarker of sperm capacitation, and subsequent acrosome reaction and sperm–egg penetration (Barbonetti et al, 2010). In crustaceans such as brachyuran species, many unique characteristics have been reported in spermatic duct, which is comparable to mammalian epididymis, including the late formation of sperm acrosome (Stewart et al, 2010), packaging of sperm within spermatophoric capsules, which are found in accordance with different types of proteoglycan secretions from the specific parts of spermatic duct (Sainte-Marie and Sainte-Marie, 1999b; Simeó et al, 2009; Zara et al, 2012; Nascimento et al, 2013). In the latter regard, it is believed that spermatophore formation is necessary process to transport sperm from male to female, since brachyuran sperm are immotile. In fact, the phenomenon takes place within the proximal and middle spermatic ducts (PSD and MSD) where PSD secretion is secreted to wrap around individual sperm and form thick wall of spermatophore (Sainte-Marie and Sainte-Marie, 1999a). Secretions of MSD are made up of neutral polysaccharides and proteins, which further impregnate onto the spermatophoric wall and/or the sperm surface (Zara et al, 2012), We addressed here the spatiotemporal acquisition of the spermatic duct secretions during membrane modification, which are considerable part of sperm maturation process that receives much less attention in crustacean species.

Alpha-2 macroglobulin (α2M) is an abundant protease inhibitor in blood circulation of vertebrates and invertebrates (Rehman et al, 2013). It belongs to the protein superfamily that shared an identical sequence of the internal thiol ester domain, GCGEQNM in several species. This thiol ester family includes murioglobulins (MuGs), ovomacroglobulins (OvMs), α1-inhibitor III (α1-I3), pregnancy zone proteins (PZPs), complement protein C3, C4 and C5, insect thiol ester protein (TEPs), CD 109, glycosylphosphatidylinositol (GPI)-anchored α2M and α2M (Chen et al, 2006). α2M was characterized in the plasma of a broad spectrum of organisms such as human, monkey, horse, birds, frog, and in hemolymph of insects and crustaceans (Ma et al, 2010).

α2M contains many *N*-linked glycosylation sites (Armstrong and Quigley, 1999) and it forms the homotetramer and homodimer of identical subunits of ~180 kDa (Feldman et al, 1985). The active isoform of α2M acts as proteinase inhibitor by entrapping any proteases in plasma without direct inactivation of the proteinase active site (Marrero et al, 2012). The major reactive sites of α2M are: 1) the bait region involved in an initial step of α2M entrapping mechanism; 2) the internal thiol ester region; 3) the receptor-binding site at the C-terminus; 4) transglutaminase reactive site; and 5) metalloprotein domain (majorly zinc protein). It is not only the protease inhibitor, but also a carrier protein for transporting numerous growth factors, cytokines and hormones in human (Borth, 1992; Rehman et al, 2013). Sequences of *α2M* are conserved in broad spectrum ranging from vertebrates to invertebrates including crustaceans (Qin et al, 2010). In crustaceans, *α2m* has been characterized only in hemocytes and reported to be involved in immune system in many crustacean species (Rattanachai et al. 2004). In reproductive system, the α2M is expressed in human seminal plasma (Glander et al, 1996; Birkenmeier et al, 1998). Its concentration significantly declines upon vasectomy suggesting its

regulation pathway from the upper reproductive tract (Glander et al, 1996). α2M is activated by cleaving off its bait region leading to the exposure of the receptor-binding domain to bind specifically to low-density lipoprotein receptor-related protein (LRP) on the proacrosomal membrane, middle piece, and tail of spermatozoa (Birkenmeier et al, 1998). In this study, we characterized α2M in epithelium of *Portunus pelagicus* reproductive tract which is secreted into MSD fluid where it associated to form spermatophoric wall as well as acquisition onto sperm plasma membrane and the acrosome. In addition, its function as a protease inhibitor was also demonstrated which partly supported its significance during sperm maturation in male reproductive tract of this crab species.

Results

Region-specific expression of SFPF-250 protein in spermatic duct and its localization on sperm surface

Sperm collected from testis, PSD, MSD, DSD and spermatheca and their corresponding fluid were analyzed by 10% SDS-PAGE and Coomassie blue staining. The results showed the major protein bands at molecular masses of ~85, 70, 55, 42, 25 and 14 kDa in all samples. Interestingly, the band at molecular masses of ~250 kDa was present exclusively in MSD fluid (Fig. 1). We therefore termed this protein "spermatic fluid specific protein" (SFSP-250) and excised it for further MALDI-TOF MS/MS analyses. The mass spectra showed 167410 masses and 122 protein scores, which contained 2 monoisotopic masses of neutral peptides with the *m/z* value of 1031.4494 and 1277.5710. The sequences of the two peptide fragments were RYMNTGYQRQ and RYTLPPNDECR, respectively. MASCOT database search revealed that both fragments were matched perfectly with *Scylla paramamosain* α2M.

In addition, the *N*-terminal amino acid sequencing identified 'NYILTTP" peptide sequence. Using NCBI Blast search tool, these amino acid sequences were 100% identical with α2M of *S. paramamosain* and *S. trituberculatus*, and similar to α2M of *Eriocheir sinensis* and *S. serrata* (67% and 50%, respectively). Therefore, we additionally assigned the name of SFPF-250 to be Ppα2M.

Bioinformatic comparison of *Ppα2m* with other existing *α2Ms*

A cDNA sequence of *Ppα2m* was obtained from the spermatic duct using specific primers designed from the sequences of *S. serrata α2m*. The partial cDNA sequence of *Ppα2m* (911 nucleotides) was obtained which could be translated into 303 amino acids. The partial cDNA sequence of *Ppα2m* was submitted to NCBI Genbank database with accession number KX688756, which showed high percent similarity with two Serrata species, *S. serrata* (92%) and *S. paramamosain* (92%). The sequence similarity to the other vertebrates and invertebrates were considerably moderate or low, suggesting the high sequence conserve within Serrata genera. Interestingly, a multiple alignment of *Ppα2m* with other *α2Ms* showed highly conserved receptor-binding domain throughout crustaceans and mammals (Fig. 2), suggesting that *α2M* would share its binding to the common receptors across many species.

Characterization of carbohydrate moieties in Ppα2M

MSD fluid proteins were separated by SDS-PAGE and lectin-blotted with various types of lectin, including ConA, WGA and GSIB4. The results showed that ConA and WGA were strongly reactive with the single 250-kDa band, while GSIB4 lectin showed only a weak

positive signal with this band (Fig. 3A). These results suggest that the 250-kDa Ppα2M protein at least contains mannose (Man) and N-acetylglucosamine (GlcNAc) residues.

The carbohydrate composition of Ppα2M was further analyzed by gas-liquid chromatography (GLC). It was shown that the major peaks corresponding to Man and GlcNAc were obtained with a proportion of 3:2 on the chromatogram (Fig. 3B). Considering binding specificity of ConA and WGA to high-Man and GlcNAc-terminated *N*-linked glycans, Ppα2M is suggested to exclusively contain Man- and GlcNAc-rich *N*-linked glycans.

Expression of *Ppα2m* in spermatic duct

We further conducted *in situ* hybridization to visualize the presence of *Ppα2m* mRNA in the entire length of spermatic duct. The result showed an intense positive signal of the *Ppα2m* antisense RNA labelling in the cytoplasm of epithelial cells in many parts of spermatic duct, from PSD to DSD (Fig. 4B-E). The positive signals were also broadly detected in the other tissues including ED + androgenic gland, female spermatical epithelium and hematopoietic tissue (Fig. 4F, G and H). Interestingly, testicular tissues did not show any positive labelling (Fig. 4A). Negative controls where MSD sections were incubated with the sense RNA probe of *Ppα2m* or muscle sections incubated with anti-sense strands showed negative staining (Fig. 4J and I, respectively). The result suggested that *Ppα2m* transcripts were present in the epithelial cells of the entire spermatic duct tissues but not in the testis.

The expression of Ppα2M was additionally revealed by Western blotting using a polyclonal antibody against human receptor-binding domain of α2M (Fig. 5). The intense immunoreactive band at 250 kDa was detected in MSD fluid protein and the staining intensity was much lower in the DSD fluid and spermathecal fluid proteins. The additional

immunoreactive band of 75 and 45 kDa with anti-α2M was also shown in the fluid proteins of the MSD as well as in the sperm protein extracts collected from PSD and MSD sperm. The two minor reactive bands of anti-α2M at 75 and 45 kDa may represent the cleaved products of Ppα2M as that reported for human seminal plasma α2M (www.abcam.com/alpha-2-macroglobulin-antibody-ab48555.html). Testicular sperm (Tes) and spermathecal sperm (Spth) showed minimal reactivity with anti-α2M antibody.

Immunolocalization of Ppα2M in the epithelial cells and sperm in the spermatic duct

The distribution of Ppα2M in the male reproductive organs was examined by indirect immunofluorescence (IIF) and confocal microscopy. In spermatic duct, an intense immunoreactivity of anti-α2M was localized at the apical surface of MSD epithelial cells and the matrix substances surrounding sperm masses as well as capsules of the spermatophores within MSD (Fig. 6A-D). There was no anti-α2M reactivity observed in epithelium and matrix substance within DSD (Fig. 6E-H). In the female spermatheca, anti-α2M reactivity could be seen only in the sperm masses where the capsules were completely expelled, while immunoreactivity of anti-α2M was not observed in spermathecal epithelium and the associated duct (Fig. 6I-L).

As Ppα2M was present in the epithelium and fluid of spermatic duct, we further investigated the dynamic of this protein on the transiting sperm surface using immunofluorescent staining. It was found that α2M was detected on the sperm plasma membrane collected from MSD sperm. A moderate immunoreactive signal was also observed inside the matrix of the acrosome (Fig. 7A and B, AC). In spermathecal sperm, some anti-α2M reactivity as a spotted staining pattern was observed along the radial arms of nucleus (RA), as well as within AC (Fig. 7C and D).

Ppα2M functioned as a protease inhibitor

The existence of Ppα2M onto the sperm surface and within the acrosomal matrix led us to further investigate whether it can act as a protease inhibitor for preventing sperm enzymes from being activated. The results in Figure 8 was clearly evident that the intact Ppα2M (linked onto nitrocellulose membrane by anti-α2M antibody to preserve its natural configuration) was able to inhibit the activity of exogenous trypsin up to 65% (panel B). The original trypsin activity was 802 and 3,152 AFU (arbitrary fluorescent units) at 15 and 120 min, respectively. They were ceased to become 163 and 1,077 AFU at the same timing points (about 3-5 fold decrease) in the case where adsorbed-α2M was included in the reaction mixture (panel A). Inhibition control in which 20 μM APMSF was included into the mixture showed a basal trypsin activity of 118 and 571 AFU at 15 and 120 min, respectively.

Discussion

Before fertilization, crustacean sperm have to undergo 3 main processes similar to those of mammals: 1) spermatogenesis and spermiogenesis where the stem cells have differentiated into structurally developing sperm cells (Stewart et al, 2010); 2) sperm maturation taking place in spermatic duct (Zara et al, 2012; Sroyraya et al, 2013), some molecular events of which were described herein 3) capacitation-like events where the sperm membrane are modified to be ready for sperm acrosome reaction in female reproductive organ, namely spermatheca (Niksirat et al, 2014; Wang et al, 2015). In *P. pelagicus*, post-testicular changes have been evident in the middle spermatic duct (MSD) where the nuclear changes and spermatophoric capsules are observed (Stewart et al, 2010). In this study, we further characterized the 250 kDa

MSD-specific protein as alpha-2 macroglobulin (Ppα2M) which was the first report in male reproductive tissue. The protein was exclusively found in MSD fluid, and rather specifically localized on apical portion of epithelium of MSD, where it secreted and absorbed onto sperm surface inside spermatophore.

Partial sequence of *Ppα2m* was cloned from cDNA of spermatic duct tissue (911 bp) and its deduced 303 amino acid sequence shared similarity with other *α2Ms* in crustaceans such as *S. serrata* (Vaseeharan et al, 2007), *S. paramamosain* (unpublished), *E. sinensis* (Qin et al, 2010) and *M. rosenbergii* (Ho et al, 2009). Crustacean *α2m* contains several conserved domain such as bait region, thioester domain and receptor-binding domain (RBD). This also holds true throughout invertebrates and vertebrates (Lin et al, 2008; Qin et al, 2010), particularly the *N*-terminally located RBD within the GGxxxTQDTVXAXXA amino acid block including GGFYSTQDTVVAMQA of Ppα2M shown in this study. *N*-linked glycosylation is also common within α2M protein that has been reported abundantly in crustaceans. Giving some examples are 12 *N*-liked glycosylation sites of *E. sinensis* (Qin et al, 2010), 11 sites for *L. vannamei* (Lin et al, 2008), and *S. serrata* (Vaseeharanet al, 2007), 8 sites for *P. monodon* (Lin et al, 2007), and *M. japonicas* (Rattanachai et al, 2004) and 6 sites for *M. rosenbergii* (Ho et al, 2009). In the case of Ppα2M, ~250 kDa MSD protein strongly reactive with ConA and WGA in the 3:2 ratio indicating the main carbohydrate compositions of Man and GlcNAc presumably on the *N*-glycosylation site of Ppα2M. Physiologically, α2M is a large glycoprotein and well-known protease inhibitor isolated from plasma serum of vertebrates, hemolymph of invertebrates and the egg white of birds and reptiles (Feldman et al, 1985; Raymond et al, 2009; Lim et al, 2011). Crustacean α2M has been reported to be synthesized from hemocytes and translocated into open-blood circulation by forming homodimeric structure (Ma et al, 2010). Likewise, *Ppα2m* mRNA

238 expression was strongly detected in hematopoietic tissue and epithelium along spermatic duct, a
239 similar manner that have been studied in invertebrates and mammals (Lim et al, 2011; Neves et
240 al, 2012). Brachyuran α2M produced from hemocytes has specific functions in protease inhibitor
241 related to immune system–its expression increases significantly after 12 h and 24 h injected with
242 bacteria and lipopolysaccharide (LPS) in *S. sinensis* and *S. serrata*, respectively (Vaseeharan et
243 al, 2007; Qin et al, 2010), as well as in humans (Feldman et al 1985; Marrero et al, 2012;
244 Rehman et al, 2013).

245 We have extrapolated the existence and localization of Ppα2M in spermatic duct, which
246 involved in sperm membrane modification in the blue swimming crab as previously reported in
247 human (Glander et al, 1996; Birkenmeier et al, 1998). *Ppα2m* mRNA was transcribed in the
248 epithelium of PSD and MSD, and then translated into a secretory protein released into the
249 adluminal compartment, particularly MSD which was finally acquired onto the plasma
250 membrane and acrosomal vesicle (AC) of MSD sperm. These results suggest that Ppα2M, as a
251 well-known protease inhibitor, may act as the enzyme masking molecules on the sperm
252 membrane and the AC during sperm transport along the MSD. In fact, its association with sperm
253 AC within MSD could be because the sperm acrosomal formation is accomplished within this
254 spermatic duct portion, leading to sperm maturation structurally and functionally within this
255 MSD (Stewart et al, 2010; Sroyraya et al, 2013). In PSD and MSD portion, acrosome of sperm
256 was formed by extension and folding of the sperm membrane between AC and nucleus to house
257 the protease into the acrosome (Stewart et al, 2010; Sun et al, 2010). Upon sperm transit into
258 female spermatheca where sperm have been kept for several weeks, sperm undergo another step
259 of morphological changes. The Ppα2M surrounding spermathecal sperm membrane was partly
260 removed whereas its association within AC matrix is unchanged.

As mentioned earlier, α2M is known to be a strong protease inhibitor existing in many systems of the organisms, particularly the circulatory system (Marrero et al., 2012; Rehman et al., 2013). We have also shown here that Ppα2M exerts its functions as a potent protease inhibitor (at least for trypsin) that is initially synthesized in the MSD epithelium and then secreted into the fluid where it is adsorbed onto sperm plasma membrane and the acrosomal matrix. We thus believe that MSD-specific Ppα2M possibly prevents the premature activation of the acrosomal enzyme (premature AR) by interacting with any proteases on the sperm membrane (in MSD) and within AC during sperm storage in spermatic duct and even sperm translocation into female spermatheca. Its existence in MSD, adsorption to modify sperm surface and inhibition of protease activity would thus considerably the first piece of evidence that signifies sperm maturation process in this crab species.

Materials and Methods

Tissue preparation for paraffin sectioning

Mature male and female blue swimming crabs, *P. pelagicus*, 150 g in body weight, were naturally caught from Thai gulf, Samutsongkram province, Thailand. They were acclimatized for 1-2 days before tissue isolation. The handing protocol of aquatic animals were followed the guidelines of Animal Ethical Practices, Australia. Briefly, the crabs were anesthetized on ice and reproductive organs, including testis, proximal spermatic duct (PSD), middle spermatic duct (MSD), distal spermatic duct (DSD), ejaculatory duct (ED) and female spermatheca, were removed and immediately fixed with Davidson's fixative solution (37% formalin, ethanol, glacial acetic acid, tap water in ratio of 2: 3: 1: 3) for 24 h. Then, the tissues were processed for paraffin embedding as previously described (Senarai et al, 2016). The

specimens were sectioned at 7 μm-thick and laid on tetramethylsilane ($Si(CH_3)_4$)-coated slides for immunofluorescent staining and *in situ* hybridization.

Collection of spermatic duct fluid and sperm protein extraction

Spermatic ducts and spermatheca were collected from male and female reproductive organs. Each part of tissues was cut into small pieces in 1-2 ml of artificial sea water (ASW: 9.3 mM $CaCl_2$, 423 mM NaCl, 9 mM KCl, 23 mM $MgCl_2$, 9.3 mM $MgSO_4$, 2.1 mM $NaHCO_3$, pH 7.8) with gentle shaking on ice. Spermatophores were separated from the fluid by centrifugation (2,000 *g*, 4°C, 10 min) while the remaining fluid were further centrifuged (12,000 *g*, 4°C, 15 min) and collected as "spermatic fluid". The sperm samples were washed extensively and homogenized in ASW containing 1:100 (v/v) PMSF (phenylmethanesulfonylfiuoride; Sigma, Saint Louis, MO). The homogenate was then centrifuged (12,000 *g*, 4°C, 15 min) and the supernatant containing sperm proteins were collected. BCA protein assay was performed to determine the protein concentration (Thermo Scientific Pierce, Rockford, IL).

Protein profiling and MALDI-TOF mass spectrometry

Approximately 10-30 μg of sperm and fluid proteins taken from testis, PSD, MSD, DSD, and spermatheca were separated by 10% SDS-PAGE under a reducing condition. For matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS/MS, the samples were alkylated by acylamide in PBS (1:20, v/w) before subjected to electrophoresis. Separated proteins were stained with 0.25% Coomassie Blue R-250 solution and destained with destaining solution (10% ethanol, 7% acetic acid). The protein band of interest (250 kDa) was cut and destained with 30% acetonitrile in 25 mM NH_4HCO_3 and then dehydrated with speedVac. Two

hundred nanograms of trypsin/Lys-C Mix and Mass Spec Grade with 0.01% of trypsin enhancer (Promega, Madison, WI) were used for in gel digestion at 37°C for overnight. Thereafter, 10-20 µl of the supernatant was collected for MALDI-TOF MS/MS analysis as previously reported (Ushijima et al, 2012). Extracted peptides were separated by reverse phase nano liquid chromatography (DiNa Map system, KYA TECH Corporation, Tokyo) and directly fractionated with MALDI-TOF spotter (DiNa Map system, KYA TECH Corporation, Tokyo) with linear gradient of solution A (2% acetonitrile, 0.1% trifluoroacetic acid, TFA) and solution B (70% acetonitrile, 0.1% TFA). Before spotting on a MALDI-TOF steel target, peptides were mixed with an equal volume with α-cyano-4-hydroxycinnamic acid (4-CHCA, Shimadzu Corporation, Kyoto, Japan) as a matrix. The internal calibration was performed using 10 pmol/µl of neurotensin, angiotensin, bradykinin and ACTH. Peptides were generated MALDI-MS and MS/MS spectrum by AB SCIEX TOF/TOF^{TM} 5800 system (AB Sciex, Redwood City, CA) with version 4.1 software (AB Sciex). MS and MS/MS spectra were analyzed by MASCOT database to search SwissProt database.

Determination of N-terminal amino acid sequences

Approximately 25 µg of MSD fluid proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was stained with CBB R-250 and destained with methanol. After drying of methanol, the 250-kDa protein band was excised and subjected to amino acid sequencer by automated Edman degradation method on the Procise 49X-HT analyzer (Applied Biosystem, Foster City, CA) as described by Takeuchi et al. (1999) (Takeuchi et al, 1999).

RNA extraction and amplification of *Ppα2m*

Total RNAs of male spermatic duct were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the instruction of manufacturer. One microgram of RNA was incubated with DNase I (Invitrogen) and used as a template for PCR using Oligo$(dT)_{12\text{-}18}$ primers and Superscript III reverse transcriptase (Invitrogen) following the manufacturer's protocol. According to the highly conserved domain of *S. serrata* and *S. paramamosain α2m* genes, the first strand cDNA was amplified by using *α2m* primers, AMGGSP Fw (5'-GCCTCAGCAGTGTGTCCTCCCGCCATCCAT-3') and AMGGSP Rw (5'-GTAGTAGTCGT ACACCACCCACTGTGCCGG-3') followed the method described by Vaseeharan et al. (2007) (Vaseeharan et al, 2007). The PCR condition was 1 cycle of 98°C for 2 min, 35 cycles of 98°C for 10 sec, 50°C for 60 s, 72°C for 60 s and 1 cycle of 72°C for 2 min. Then, PCR products were analyzed by 1% agarose gel electrophoresis and purified by gel and PCR clean-up system (Promaga, Madison, WI). pGEM T-easy vector (Promega) was ligated to clone PCR products by a Ligation High Version2 (Toyobo, Osaka, Japan) and transformed into DH5α competent cells. The DNA-inserted plasmids were purified following previously reported (Garénaux et al, 2015) and precipitated by 20% of polyethylene glycol (PEG). pGEM T-easy vector containing *P. pelagicus α2m* gene was amplified by forward and reverse primers of M13 promoter. PCR products were precipitated with 3 M sodium acetate and 95% ethanol, respectively, then dissolved with hidihormaldehyde as previous report (Garénaux et al, 2015). The sequences were analyzed by Applied Biosystems 3500 Series Genetic Analyzers (Applied Biosystems, Foster City, California).

Gas liquid chromatography (GLC)

Ten micrograms of MSD fluid proteins mixed with galactitol (Gal-ol) as internal control were dissolved with 0.5 mol/l of hydrochloric acid methanolic solution at 65°C for 16 h. Hexane extraction was performed and the methanol phase was evaporated and re-acetylated by 0.5 ml of methanol, 10 µl of pyridine and 50 µl of acetic anhydrite. For trimethylsilyl (TMS)-derivatization, sample mixture was derivatized with TMS chloride by adding 50 µl of pyridine, 10 µl of hexamethyldisilazane (HMDS), and 5 µl of TMS chloride, and then the mixture was mixed and incubated for 30 min at RT. The mixture was dried up with N_2, diluted with 50 µl of hexane and quantitated by GLC on a capillary column (CBJ5, 0.32 mm×30 m, Shimada, Japan) at 190-260°C at 4°C/min. GLC peaks of sugar samples were compared with peak multiplicity of various derivatives of standards and calculated with sugar ratio to predict carbohydrate structure as described previously (Funakoshi et al, 1997).

Lectins and Western blottings

Approximately 10 µg of MSD fluid proteins were loaded and separated by 10% SDS-PAGE under a reducing condition and transferred onto PVDF membrane. The membrane was blocked non-specific binding with 1% bovine albumin serum (BSA) in 10 mM sodium phosphate buffer containing 150 mM NaCl (PBS), pH 7.4 for 1.5 h at RT. Then, the membranes were incubated with Concanavalin A (ConA), Wheat germ agglutinin (WGA) and Griffonia simplicifolia-IB4 (GSIB4) solutions (1 mg/µl, 1:1000 dilution) (Sigma, Saint Louis, MO) for overnight at 4°C. After washing, the membrane were probed with anti-ConA-rabbit IgG (1:1000 dilution), anti-WGA-mouse IgG (1:1500 dilution) and GSIB4 streptavidin (1:1000 dilution) (Sigma) for 2 h at RT. The membranes were washed and incubated with the corresponding secondary horse radish peroxidase (HSP) conjugated antibodies, (1:2500-1:5000 dilution) or

376 POD-streptavidin (1:5000 dilution), respectively. Then, signals were detected with an enhance
377 chemiluminescent detection reagent (ECL; Amersham Biosciences, Little Chalfont,
378 Buckinghamshire). For Western blotting analysis, 10 µg of sperm and fluid proteins were
379 separated by SDS-PAGE and transferred onto PVDF membrane. The membrane was incubated
380 in blocking solution (2.5% BSA with 0.1% of Tween 20 in PBS ($PBST_{w-20}$) for 2 h at RT. The
381 membrane was probed with polyclonal antibody against α2M-rabbit IgG (1:1000 dilution) for
382 overnight at 4°C and anti-rabbit IgG (1:5000 dilution) (Cell signaling; Santa Cruz, CA) for 2 h at
383 RT as primary and secondary antibody, respectively. Then signals were detected with ECL.
384
385 **Immunofluorescence staining**
386 Spermatophores taken from PSD, MSD and spermatheca were isolated by
387 centrifugation at 2000 *g* for 10 min, 4°C and washed several times with ASW. After gentle
388 homogenization, the mixture was filtrated through 225-µm sieve and the sperm were smeared on
389 glass slides prior to staining. In addition, tissue sections of male reproductive organs and female
390 spermatheca were deparaffiinized in xylene and rehydrated in serial ethanol series (100%, 95%,
391 80% and 70%). The smeared sperm on the glass slides and the paraffin sections were
392 permeabilized in 0.5% $PBST_{x-100}$ for 15 min and non-specific binding were blocked by 4% BSA
393 in PBS for 2 h at RT. Then, the samples were incubated with polyclonal antibody against α2M-
394 rabbit IgG (1:1000 dilution) (Cell Signaling, Santa Cruz, CA) for overnight at 4°C and anti-
395 rabbit IgG-Alexa 488 (1:1000 dilution) (Cell Signaling) as primary and secondary antibodies,
396 respectively. Nuclear staining was performed by DAPI (1:1000 dilution) (Cell signaling).
397 Immuno-reactive signals were observed under Olympus FV1000-D confocal laser scanning
398 microscope with an IX81 microscopy.

399

400 **In situ hybridization**

401 The digoxigenin (DIG) labeled *Ppα2m* mRNA probe was produced using DIG RNA
402 labeling kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany). The 7-μm thick male
403 reproductive tissues on glass slides were deparaffinized, rehydrated, and treated with 50 μg/ml
404 protease K at 37°C for 30 min. Then, the tissue sections were covered with 250 μl of 4%
405 paraformaldehyde at 4°C for 10 min and incubated with prehybridization buffer (4× SSC and
406 50% (v/v) formamide) for post-fixing and blocking the non-specific binding, respectively. For
407 hybridization, DIG-labeled *Ppα2m* mRNA probes were diluted in DIG Easy hybridization buffer
408 (1:250 dilution) and overlaid the sections at 42°C for overnight. After washing with 2× SSC and
409 1× SSC 2 times for 10 min/each, the sections were incubated with NTE buffer (0.5 M NaCl, 10
410 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 37°C for 30 min. Following the manufacturer's protocol
411 of DIG nucleic acid detection kit (Roche Diagnostics), the sections were incubated with blocking
412 solution at 37°C for 1 h and alkaline phosphatase conjugated anti-DIG antibody, anti-DIG-AP
413 (1:5000 dilution) for 1 h at 37°C. Finally, the signals were developed with NBT/BCIP solution
414 and observed under light microscopy (Olympus DP70).

415

416 **Protease inhibitor assay of Ppα2M using fluorogenic substrates**

417 We prepared native Ppα2M (known to function in the dimerized forms) that conjugated
418 to its corresponding antibody on the nitrocellulose membrane for testing the function of Ppα2M
419 proteins. Approximately 2 μg of 1 mg/ml rabbit polyclonal antibody against human α2M was
420 adsorbed onto a nitrocellulose membrane (Millipore, Bedford, MA) and blocked its non-specific
421 binding with 1% BSA in PBS for 2 h. Semi-purified MSD fluid (through a centrifugal device

with a cut-off of 100 kDa) was used to conjugate with the membrane linked antibody (overnight, 4°C). The excessive MSD proteins were washed away by immersing in PBS with gentle agitation. The membrane was cut into 4×4 mm size before putting into the bottom of a 96-well plate. The reaction mixture included trypsin (0.001 unit activity) in the 90 µl reaction buffer (10 mM $CaCl_2$, 0.001% Triton X-100 in 50 mM Tris-HCl, pH 7.5) and left in room temperature for 1.5 h. The mixture was transferred into a black 96-well flat bottom plate (Corning Incorporated, Corning, NY, USA) and mixed with 10 µl of 10 µM trypsin specific substrates (Boc-Gln-Ala-Arg-MCA; Peptides International, Louisville, KY, USA). The released free 7-amino-4-methylcoumarin (AMC) was spectrofluorometrically measured at various time points using a Spectra Max Gemini XS (Molecular Dynamics, Sunnydale, CA, USA) with excitation and emission wavelengths of 355 and 460 nm, respectively. Other experimental groups also included membranes dotted with anti-α2M-IgG which were further incubated with 1) PBS (as a blank control), 2) trypsin (0.001 unit activity; as positive control), 3) trypsin (0.001 unit activity) + p-amidinophynylmethylsulfonylfluoride (APMSF; 0.1 nM) and measured the release of fluorescent substrate as mentioned above.

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We thank Prof. Naoyuki Yamamoto (Nagoya University), Dr. Sirilug Magerd and Dr. Jirawat Saetan for their technical and instrumental suggestions.

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555

556 **Figure legends**

557 **Figure 1** Profiling of *P. pelagicus* sperm and fluid proteins (10 μg) separated by 10% SDS-
558 PAGE and Coomassie blue staining. Note the specific expression of a 250 kDa protein band
559 (arrowhead) in the MSD fluid. Tes, Testis; PSD, proximal spermatic duct; MSD, middle
560 spermatic duct; DSD, distal spermatic duct; Spth, female spermatheca.

561

562 **Figure 2** Multiple alignments of deduced amino acid sequences of *P. pelagicus α2m* (*Ppα2m*)
563 and other *α2Ms*. The partial sequence of *Ppα2m* was submitted to NCBI Genbank database with
564 accession number KX688756. The receptor binding domain (RBD) of *Ppα2m* is aligned with
565 *Homo sapiens* (GenBank: AAT02228.1), *Mus musculus* (GenBank: AAH72642.1), *Bos mutus*
566 (GenBank: ELR5448.1), *Danio rerio* (GenBank: NP_001188334.2), *Xenopus tropicalis*
567 (GenBank: AAH93458.1), *Limulus polyphems* (GenBank: BAA19844.1), *Pacifastacus*
568 *leniusculus* (GenBank: AEC50080.1), *Macrobrachium rosenbergii* (GenBank: ABK60046.1),
569 *Scylla paramamosain* (GenBank: CCW43201.1), *Eriocheir sinensis* (GenBank: ADD71943.1)
570 and *Scylla serrata* (GenBank: ABD61456.1). The asterisks show the positions shared the
571 identical amino acid residues that are highly conserved at the position 41-52 (in the boxed area).
572 *N*-glycosylation site in RBD of Ppα2M is conserved among brachyuran species at the position
573 103-105 (underlines), A colon (:) and a bullet (.) stand for high and low levels of amino acid
574 similarities, respectively.

575

576 **Figure 3** Carbohydrate analysis of a 250 kDa MSD fluid protein by lectin blotting and gas-liquid
577 chromatography. The resolved, blotted proteins were probed with lectins: Concanavalin A
578 (ConA), Wheat germ agglutinin (WGA), and Griffonia Simplicifolia-IB4 (GSIB4) (panel A).

The arrow indicates the 250 kDa protein with lectin reactivity. In chromatogram (panel B), the peaks with retention time of 25.6 min (arrow) and 32.85 min (arrowhead) represent Man and GlcNAc, respectively. The peak at retention time 29.7 min illustrates Gal-ol included as an internal control. The ordinate represents arbitrary unit. Numbers along the abscissa indicate the retention time.

Figure 4 Expression of *Ppα2m* mRNA in the male reproductive organs including testis, spermatic duct and androgenic gland, hematopoietic tissue and muscle. Note the intense staining in the cytoplasm of epithelial cells (ep) lining early and distal portions of the proximal spermatic duct, early PSD and distal PSD (B and C), MSD (D), DSD (E), ED and the overlying androgenic gland (AG) (F), female spermatica (G) and hematopoietic tissue (H). Testis (A) shows negative staining similar to those of negative controls which are MSD section probed with a sense strand Ppα2M (J) or muscle section probed with anti-sense strand (I). sc, spermatocyte; sm, sperm mass; Sph, spermatophore; ma, matrix; mu, muscle; hc, hematocyte.

Figure 5 Western blotting of sperm and fluid proteins with anti-α2M polyclonal antibody. Sperm protein extracts and the corresponding fluid proteins were collected from testis (Tes), proximal-(PSD), middle-(MSD), distal-spermatic duct (DSD), spermatheca (Spth). Arrowheads indicate an immunoreactivity at 250, 75 and 45 kDa protein bands.

Figure 6 Immunofluorescent localization of Ppα2M in epithelium of spermatic duct. The strong fluorescent signals anti-α2M is apparent in the epical cytoplasm of MSD epithelial cells (a-d, arrowheads), spermatophoric (Sph) capsule and sperm masses (b, c, asterisks), whereas it has no

602 signal of α2M in epithelial cells of DSD (e-h) and spermatheca (i-l). The tissue were
603 counterstaining with DNA staining dye, DAPI (red). The corresponding DIC micrographs are
604 shown in the right-most column. Bars: 100 μm. ep, epithelium; ma, matrix; sm, sperm mass.
605
606 **Figure 7** Surface localization of Ppα2M on the isolated sperm from various parts of spermatic
607 duct and female spermatheca. MSD sperm showed highly intense fluorescent signals on the
608 sperm surface (arrowheads) and a weak fluorescent in the acrosome (AC, asterisk) (a, b). The
609 surface staining a rubbly reduced in the spermathecal sperm, mostly on the radial nuclear arms
610 (RA) and remaining in AC (c, d). N, nucleus.
611
612 **Figure 8** Ppα2M served as a potent protease inhibitor towards trypsin activity. Natural form of
613 Ppα2M was conjugated to the anti-α2M antibody that had been adsorbed onto nitrocellulose
614 membrane before testing with the purified porcine trypsin (-×-). The enzyme activity was
615 measured by monitoring the released fluorescent AMC from Boc-Gln-Ala-Arg-MCA for 2 h and
616 expressed as mean of arbitrary fluorescent units obtained in triplicate experiments (A) and the
617 percent activity of trypsin at 2 h (B). The bars stand for standard deviations.
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Figure 1

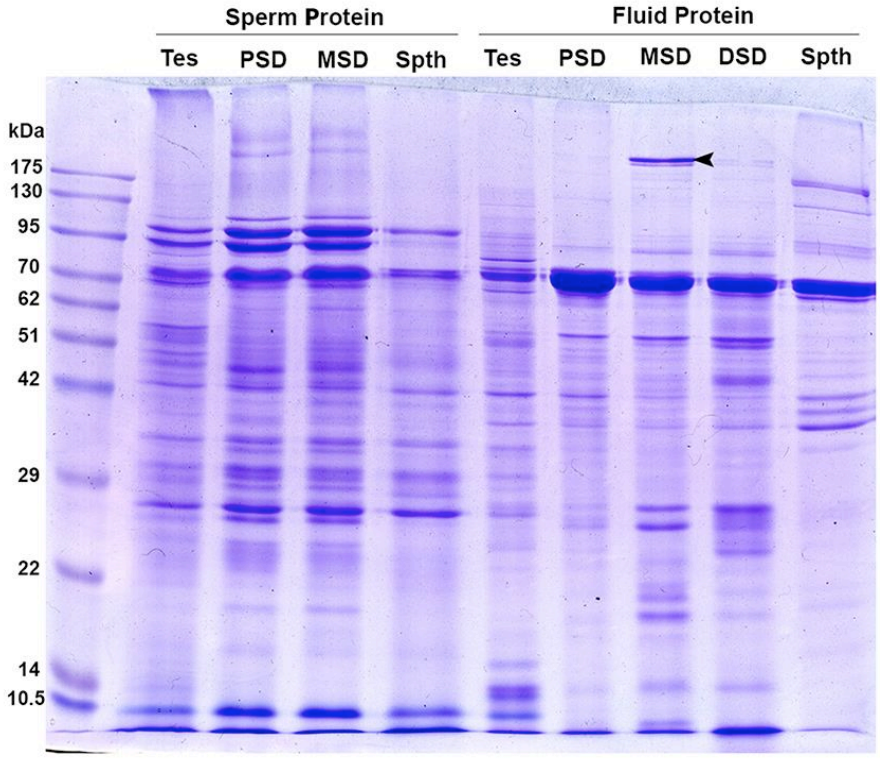
Sperm Protein
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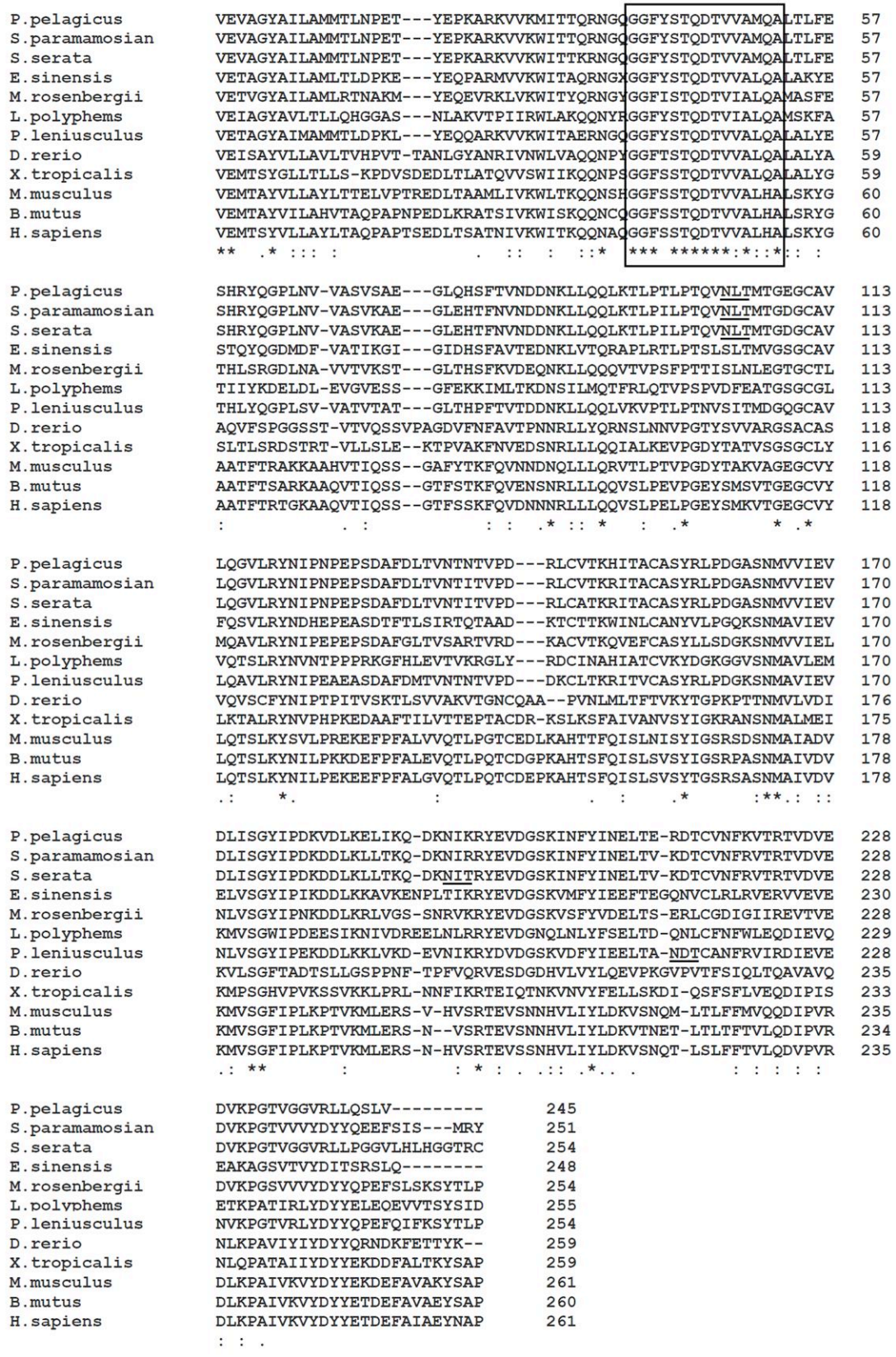
Figure 2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E.sinensis VETAGYAILAMLTLDPKE---YEQPARMVVKWITAQRNGXGGFYSTQDTVVALQALAKYE 57
M.rosenbergii VETVGYAILAMLRTNAKM---YEQEVRKLVKWITYQRNGYGGFISTQDTVIALQAMASFE 57
L.polyphems VEIAGYAVLTLLQHGGAS---NLAKVTPIIRWLAKQQNYRGGFYSTQDTVIALQAMSKFA 57
P.leniusculus VETAGYAIMAMMTLDPKL---YEQQARKVVKWITAERNGQGGFYSTQDTVVALQALALYE 57
D.rerio VEISAYVLLAVLTVHPVT-TANLGYANRIVNWLVAQQNPYGGFTSTQDTVVALQALALYA 59
X.tropicalis VEMTSYGLLTLLS-KPDVSDEDLTLATQVVSWIIKQQNPSGGFSSTQDTVVALQALALYG 59
M.musculus VEMTAYVLLAYLTTELVPTREDLTAAMLIVKWLTKQQNSHGGFSSTQDTVVALHALSKYG 60
B.mutus VEMTAYVILAHVTAQPAPNPEDLKRATSIVKWISKQQNCQGGFSSTQDTVVALHALSRYG 60
H.sapiens VEMTSYVLLAYLTAQPAPTSEDLTSATNIVKWITKQQNAQGGFSSTQDTVVALHALSKYG 60
** .* ::: : . :: : ::* *** ******:*::*:: :
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S.paramamosian SHRYQGPLNV-VASVKAE---GLEHTFNVNDDNKLLQQLKTLPILPTQVNLTMTGDGCAV 113
S.serata SHRYQGPLNV-VASVKAE---GLEHTFNVNDDNKLLQQLKTLPILPTQVNLTMTGDGCAV 113
E.sinensis STQYQGDMDF-VATIKGI---GIDHSFAVTEDNKLVTQRAPLRTLPTSLSLTMVGSGCAV 113
M.rosenbergii THLSRGDLNA-VVTVKST---GLTHSFKVDEQNKLLQQQVTVPSFPTTISLNLEGTGCTL 113
L.polyphems TIIYKDELDL-EVGVESS---GFEKKIMLTKDNSILMQTFRLQTVPSPVDFEATGSGCGL 113
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D.rerio AQVFSPGGSST-VTVQSSVPAGDVFNFAVTPNNRLLYQRNSLNNVPGTYSVVARGSACAS 118
X.tropicalis SLTLSRDSTRT-VLLSLE--KTPVAKFNVEDSNRLLLQQIALKEVPGDYTATVSGSGCLY 116
M.musculus AATFTRAKKAAHVTIQSS--GAFYTKFQVNNDNQLLLQRVTLPTVPGDYTAKVAGEGCVY 118
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H.sapiens AATFTRTGKAAQVTIQSS--GTFSSKFQVDNNNRLLLQQVSLPELPGEYSMKVTGEGCVY 118
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P.pelagicus LQGVLRYNIPNPEPSDAFDLTVNTNTVPD---RLCVTKHITACASYRLPDGASNMVVIEV 170
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H.sapiens LQTSLKYNILPEKEEFPFALGVQTLPQTCDEPKAHTSFQISLSVSYTGSRSASNMAIVDV 178
.: *. : . : .* :**.: ::
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H.sapiens KMVSGFIPLKPTVKMLERS-N-HVSRTEVSSNHVLIYLDKVSNQT-LSLFFTVLQDVPVR 235
.: ** : : * : . .:: .*.. . : : : : :
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S.paramamosian DVKPGTVVVYDYYQEEFSIS---MRY 251
S.serata DVKPGTVGGVRLLPGGVLHLHGGTRC 254
E.sinensis EAKAGSVTVYDITSRSLQ-------- 248
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X.tropicalis NLQPATAIIYDYYEKDDFALTKYSAP 259
M.musculus DLKPAIVKVYDYYEKDEFAVAKYSAP 261
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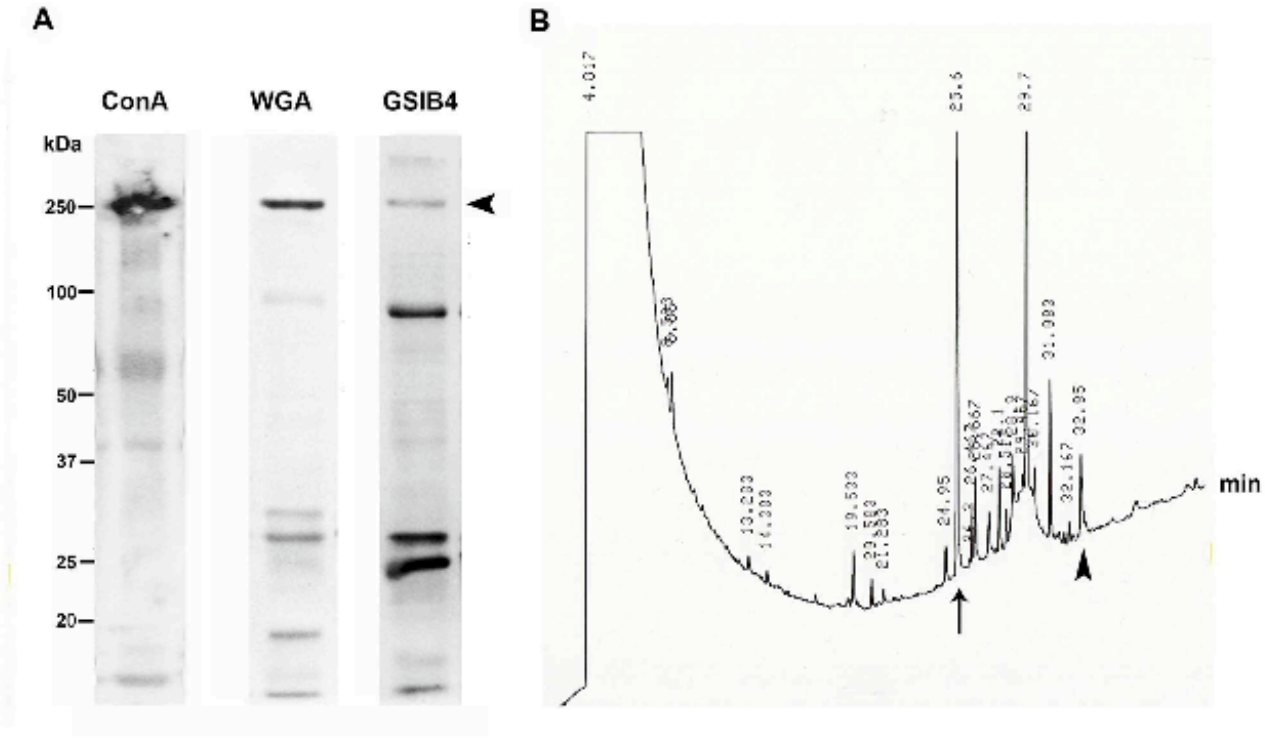
Figure 3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ConA
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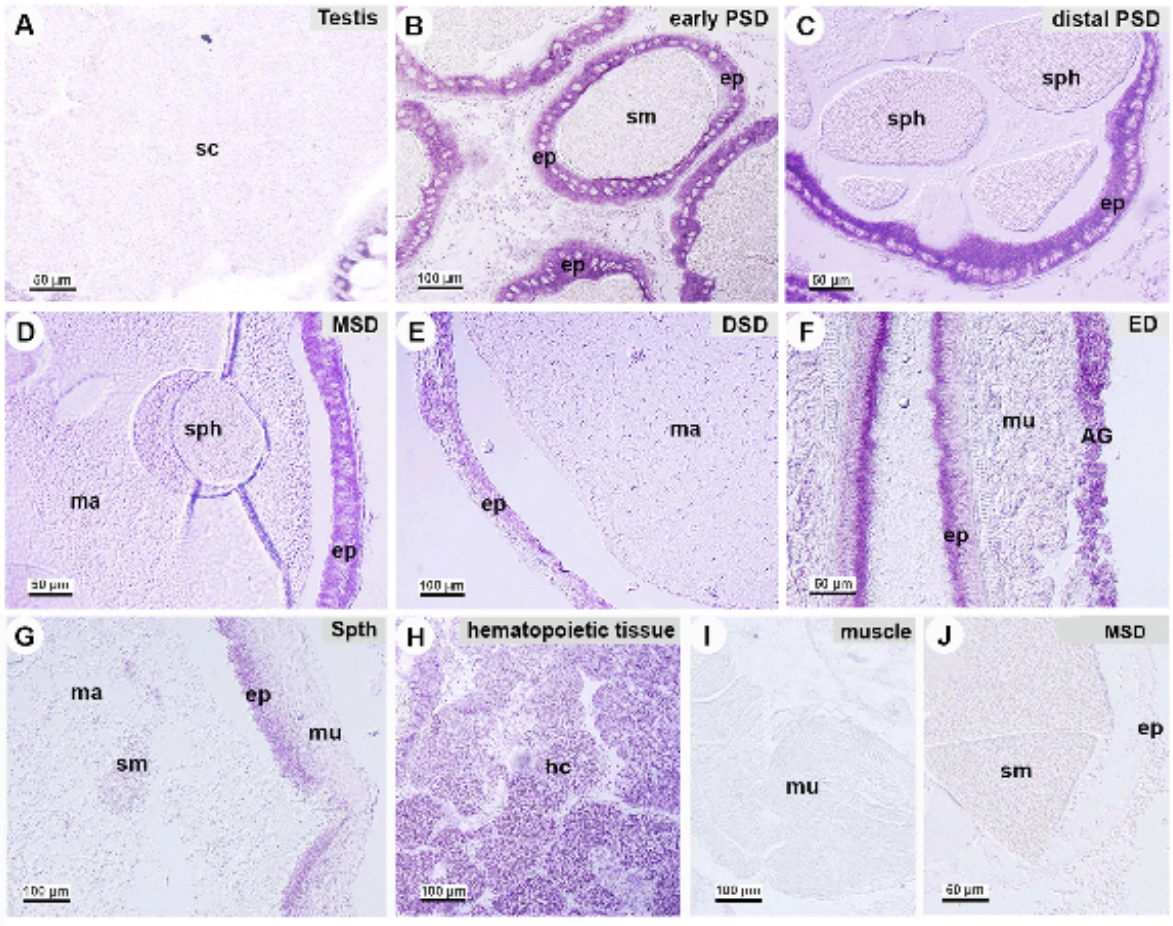
Figure 4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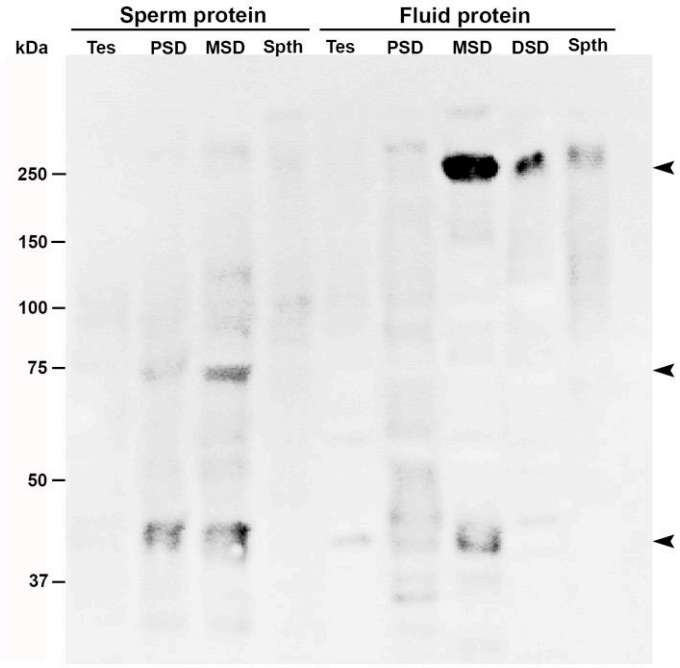
50 µm
G
Spth
ma
ep
mu
sm
100 µm
H
hematopoietic tissue
hc
100 µm
I
muscle
mu
100 µm
J
MSD
ep
sm
50 µm

Figure 5


Sperm protein
Fluid protein
kDa
Tes
PSD
MSD
Spth
Tes
PSD
MSD
DSD
Spth
250
150
100
75
50
37

648

Figure 6

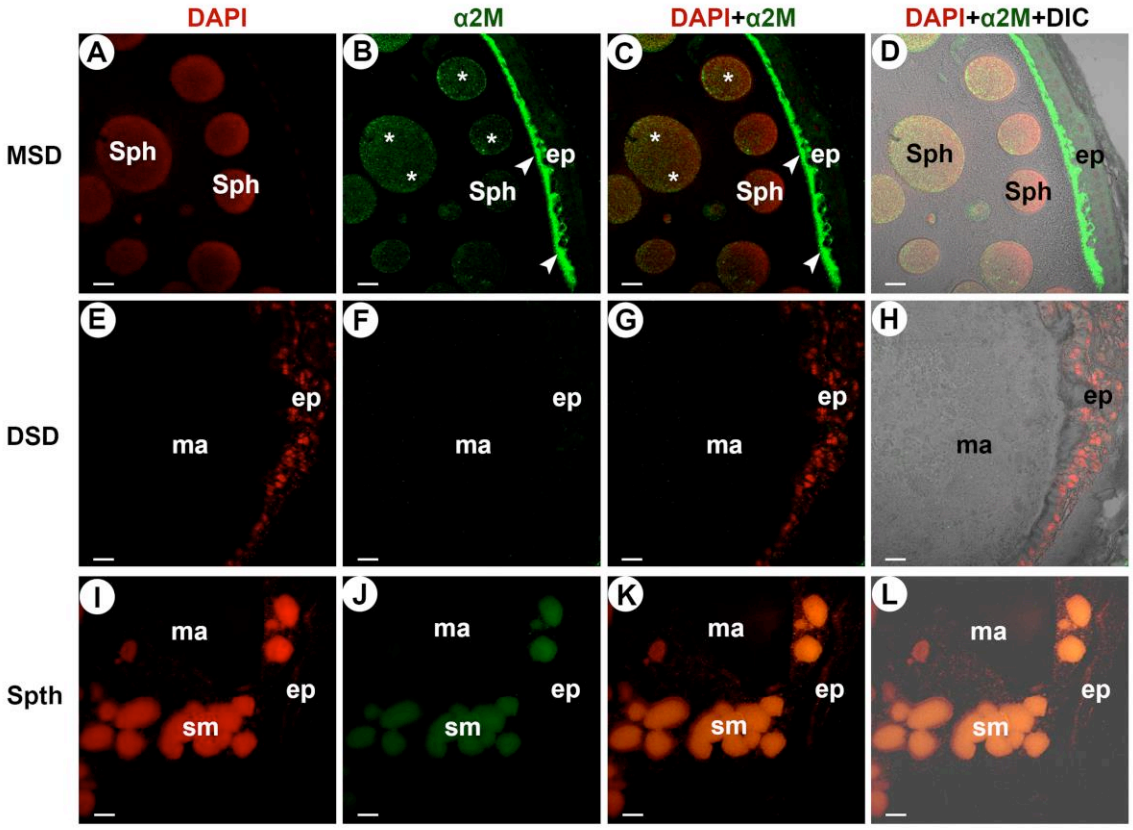

DAPI
α2M
DAPI+α2M
DAPI+α2M+DIC
MSD
DSD
Spth
A
B
C
D
E
F
G
H
I
J
K
L
Sph
ep
ma
sm

Figure 7

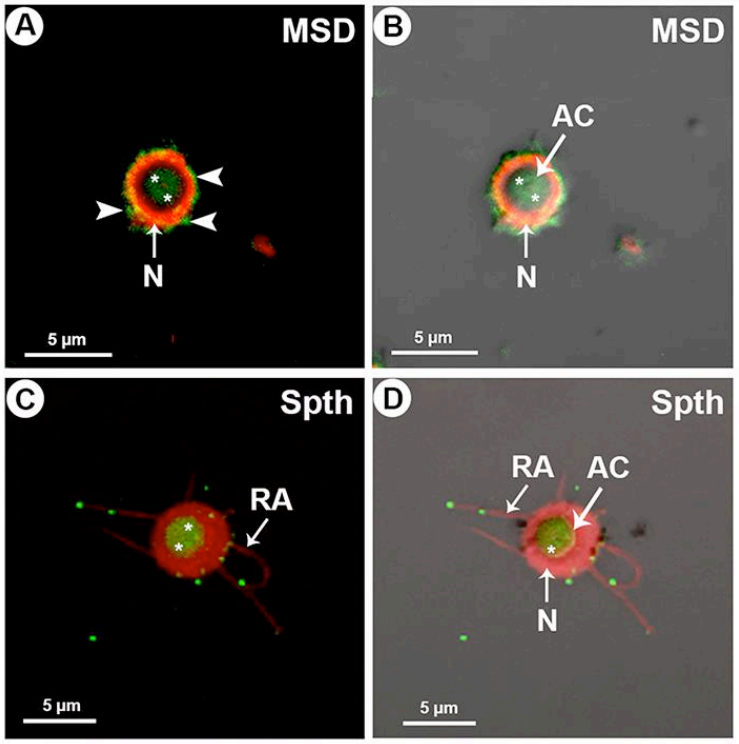
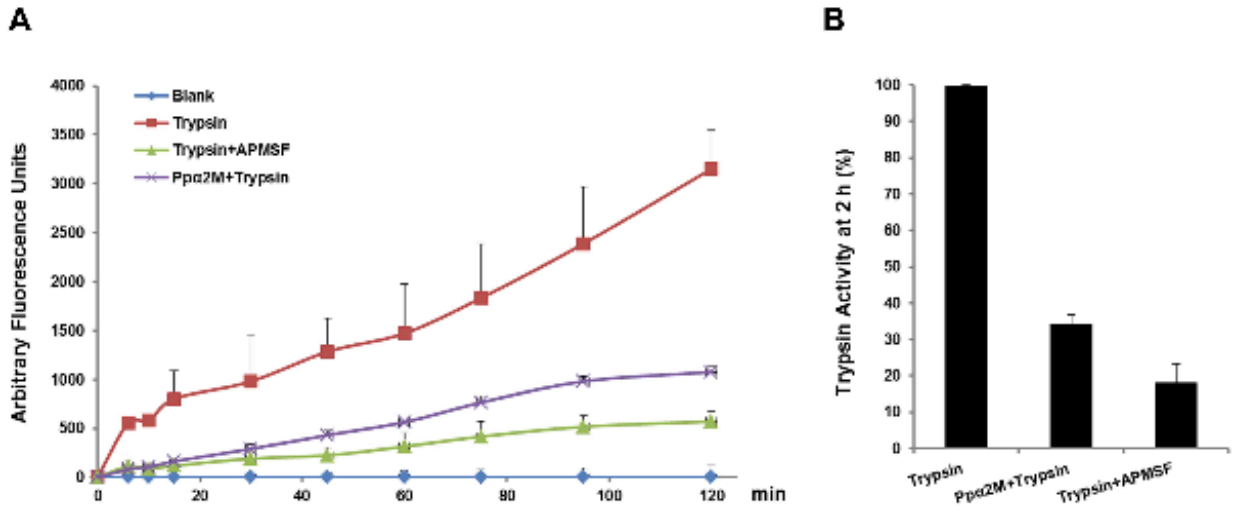


Figure 8


A
B
Arbitrary Fluorescence Units
4000
3500
3000
2500
2000
1500
1000
500
0
Blank
Trypsin
Trypsin+APMSF
Pp⍺2M+Trypsin
0
20
40
60
80
100
120
min
Trypsin Activity at 2 h (%)
100
90
80
70
60
50
40
30
20
10
0
Trypsin
Pp⍺2M+Trypsin
Trypsin+APMSF