Cathepsin A protein from the accessory sex gland of the Chinese mitten crab (Eriocheir sinensis) plays a key role in spermatophore digestion

Juan Wang \textsuperscript{a,1}, Di-An Fang \textsuperscript{b,1}, Yang Wang \textsuperscript{a,1}, Yuan-Li Wang \textsuperscript{a}, Lin Cheng \textsuperscript{a}, Lin He \textsuperscript{a}, Qun Wang \textsuperscript{a,*}

\textsuperscript{a} School of Life Science, East China Normal University, 3663 North Zhongshan Road, Shanghai 200062, China
\textsuperscript{b} Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, 9 Shanshui Road, Wuxi 214081, China

1. Introduction

In most decapods, the spermatozoa are enveloped as spermato- phores in the male seminal vesicles. During the mating period, the spermatozoa of brachyura are transferred to the spermatheca of the female and gradually broken down to release free spermatozoa (Blaxter and Southward, 1993; Hinsch, 1986). Spermatozoa consist mainly of the spermatoaphore wall, spermatoaphore matrix, and sperm groups. The spermatoaphore wall in decapods is generally composed of layers (1–3), which include acid mucopolysaccha- ride proteins, chitinase, and phenolic compounds as the main components (Uma and Subramoniam, 1961). However, further studies revealed that low molecular weight secretions during female ovulation and enzymes secreted by the glandular epithelial cells of the female spermatheca may in- volve certain unknown proteins that break down the spermato- phore wall (Diesel, 1989; Dudenhauen and Talbot, 1983).

Although the spermatoaphore breakdown and the subsequent release of free spermatozoa are necessary conditions for natural and artificial fertilization, the mechanism of spermatoaphore rup- ture in decapods remains unclear. Until now, mechanical homoge- nization and enzymatic digestion have been reported to result in free spermatozoa (Leung-Trujillo and Lawrence, 1985; Lezcano et al., 2004), but these two methods are likely to cause damage to spermatozoa and affect experimental results. Recently, our group has reported that ASG secretory proteins can effectively break down the spermatoaphore wall (Hou et al., 2010), but the target enzyme has not been identified. Therefore, further research on the function of the ASG for spermatoaphore breakdown in decapods could improve the analysis of crustacean spermatozoa qualities and crustacean reproductivity.

Accessory glands are important components of the animal male reproductive system: for example, they play an important role in mammals in improving the fertility of caudal epididymal sperm during male reproduction. Previous studies have shown that secretions from the epididymal epithelium are likely to affect sperm maturation (Dacheux et al., 2003). Recent research has suggested that ASG secretions in Holstein cattle participate in key spermatic events, including protection, motility, capacitation, acrosome reaction and sperm–oocyte interactions (Moura et al., 2007).

The Chinese mitten crab is one of the commercially most impor- tant aquaculture species in China (He et al., 2012). Although the reproductive biology of Eriocheir sinensis has received increasing attention in recent years (Fan et al., 2007; Kalinina et al., 2008;
Ma et al., 2006), very little information on the function of the ASG is available in the literature. During the later developmental stages of E. sinensis, the ASG rapidly develops dendritic branches at the junction of the seminal vesicles and ejaculatory duct. Its lumen diameter increases, which contains large amount of milky secretions (Du et al., 1988a). Why and how this phenomenon occurs remains unknown.

We hypothesized that there may be a link between ASG secretions and spermatophore wall rupture, and postulated the existence of enzymes that digest spermatophores. Despite the established function of the ASG secretory proteins, the pivotal protein function in spermatophore wall rupture has not been established. In order to clearly investigate which proteins participate in the breakdown of the spermatophore wall, we describe here the purification and characterization of ASG secretory proteins involved in spermatophore digestion, including the changes in spermatophore wall that occur over time. We identified one of the most important proteins, cathepsin A (cathA), and its role in spermatophore wall rupture. The activity of cathA during the reproductive cycle of the crab was analyzed.

2. Material and methods

2.1. Sample preparation

Healthy adult 2-year old male E. sinensis (150–200 g) with intact ASGs were purchased from the Tongchuan Road aquatic market in Shanghai, China at an early stage (in August) and at the peak stage (in November) of reproduction. Crabs were placed in an ice bath (4–6 min) until they were lightly anesthetized. ASGs were collected monthly from August to December (Du et al., 1988a), immediately removed and frozen in liquid nitrogen, and stored in −80 °C for further analysis.

2.2. Chemicals

DEAE-Sepharose, Sephacryl S-200 HR and Sephadex G-25 S were purchased from GE Healthcare (Piscataway, NJ, USA). E-64 and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Protein marker for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was from TaKaRa Biotech, Dalian, China. All other reagents were analytical grade.

2.3. Purification of ASG proteins

All purification processes were carried out at low temperatures (0–4 °C). The mature ASGs taken in November were chosen for the preparation of the ASG secretory protein extract. E. sinensis ASGs (30 g, eight male crabs) were washed and punctured by a dissecting needle in 180 ml ice-cold 25 mM phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM E-64, using a homogenizer. After the secretions had been released, the ASGs were homogenized. The activity of cathA during the reproductive cycle of the crab was analyzed.

In order to establish which ASG protein could digest the spermatophore wall, we removed spermatophores from male crab seminal vesicles (approximately 5 g, two male crabs) and put them into 100 ml of Ca+-FASW, containing 0.4 M NaCl, 15 mM KCl, 8.6 mM boric acid, 4.8 mM NaOH, and 41 mM MgSO4 (pH 7.4). We then transferred 1 ml of the middle aqueous layer to a centrifuge tube. After centrifugation at 400g for 10 min, the spermatophores were precipitated and resuspended (Hou et al., 2010; Ma et al., 2006). Based on our previous experiments, the spermatophore suspensions were individually combined with the desalted ASG secretory proteins from each peak (I–IV, II-1, II-2), the same buffer (Ca+-FASW) of the suspensions, and were then incubated at 37 °C to assay the lytic properties of the ASG. The protein capable of spermatophore wall rupture was further analyzed and identified. The spermatophores undergoing digestion were observed under a light microscope (Leica, Germany), and images were taken at different incubation times.

2.4. Protein concentration assay

Protein concentration was determined by measuring the sample solution absorbance at 280 nm, during column chromatography or by using the BCA protein assay (Smith et al., 1985), according to the manufacturer’s instructions. Bovine serum albumin (BSA) was used as the standard.

2.5. Spermatophore digestion

In order to establish which ASG protein could digest the spermatophore wall, we collected spermatophores from male crab seminal vesicles (approximately 5 g, two male crabs) and put them into 100 ml of Ca+-FASW, containing 0.4 M NaCl, 15 mM KCl, 8.6 mM boric acid, 4.8 mM NaOH, and 41 mM MgSO4 (pH 7.4). We then transferred 1 ml of the middle aqueous layer to a centrifuge tube. After centrifugation at 400g for 10 min, the spermatophores were precipitated and resuspended (Hou et al., 2010; Ma et al., 2006). Based on our previous experiments, the spermatophore suspensions were individually combined with the desalted ASG secretory proteins from each peak (I–IV, II-1, II-2), the same buffer (Ca+-FASW) of the suspensions, and were then incubated at 37 °C to assay the lytic properties of the ASG. The protein capable of spermatophore wall rupture was further analyzed and identified. The spermatophores undergoing digestion were observed under a light microscope (Leica, Germany), and images were taken at different incubation times.

2.6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Equal amounts (10 µg) of each protein in SDS–PAGE were used to determine the purity and molecular mass of the purified ASG secretory proteins, according to the method of Laemmli (1970) using 12% separating gel and 4% stacking gel. After gel electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB) in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

2.7. In-gel trypsin digestion

A gel piece containing the protein with the ability to digest spermatophores (peak II-2) was cut from the SDS–PAGE and treated with 30% ACN (100 mM NH4HCO3) until the gel was destained. After the gel was dried in a vacuum centrifuge, the in-gel proteins were reduced with dithiothreitol (10 mM DTT/100 mM NH4HCO3) for 30 min at 56 °C, and then alkylated with iodoacetamide (50 mM HCO3–/C0 to assay the lytic properties of the ASG. The protein capable of spermatophore wall rupture was further analyzed and identified. The spermatophores undergoing digestion were observed under a light microscope (Leica, Germany), and images were taken at different incubation times.
IAA/100 mM \( \text{NH}_4\text{HCO}_3 \) in the dark at room temperature for 30 min. Before the gel pieces were digested overnight in 12.5 ng/ml trypsin in 25 mM \( \text{NH}_4\text{HCO}_3 \), they were briefly rinsed with 100 mM \( \text{NH}_4\text{HCO}_3 \) and ACN, respectively. Finally, the peptides were extracted three times with 60% ACN/0.1% TFA; extracts were pooled and dried in a vacuum centrifuge.

2.8. HPLC-ESI-MS/MS shotgun analysis

Following the digestions described in Section 2.7, an EtanTM MDLC system (GE Healthcare, Piscataway, NJ, USA) was used for desalting and separation of tryptic peptides mixtures. In this system, digests were pre-concentrated at a flow rate of 20 \( \mu \text{l/min} \) on RP trap columns (Zorbax 300 SB C18, Agilent Technologies, Palo Alto, CA, USA) connected in front of an RP column (150 \( \mu \text{m id}, 100 \text{mm length, Column technology Inc., Fremont, CA, USA}). Mobile phase A (0.1% formic acid in HPLC-grade water) and mobile phase B (0.1% formic acid in acetonitrile) were selected. Then, 20 \( \mu \text{g} \) of tryptic peptide mixtures was loaded into the columns, and separation was carried out at a flow rate at 2 \( \mu \text{l/min} \) using a linear gradient of 4–50% B for 60 min. A FinniganTM LTQTM linear ion trap MS (Thermo Electron) equipped with an electrospray interface was connected to the LC setup for eluted peptide detection. Data-dependent MS/MS spectra were obtained simultaneously. Each scan cycle consisted of one full MS scan in profile mode followed by five MS/MS scans in centroid mode with the following Dynamic ExclusionTM settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s. Each sample was analyzed in triplicates.

2.9. Protein identification

Obtained MS/MS spectra were automatically searched by using BioworksBrowser rev. 3.1 (Thermo Electron, San Jose, CA, USA). The transcriptomic profiles of tests and ASG databases from E. sinensis were used for protein identification. Peptides were constrained to be tryptic and up to two missed cleavages. Carbamidomethylation of cysteines was treated as a fixed modification, whereas oxidation of methionine residues was considered as a variable modification. Protein identification results were extracted from SEQUEST out files with BuildSummary (Dai et al., 2005). The mass tolerances allowed were 2.0 Da for the precursor ions and 0.8 Da for fragment ions, respectively. The protein identification criteria from pros and cons were based on Delta CN (>0.1), peptide FDR < 0.01 and protein FDR < 0.01.

2.10. Western blot analysis

To further demonstrate the result from the HPLC-ESI-MS/MS shotgun analysis, a Western blot was performed according to Towbin’s method (Towbin et al., 1979) using a transblot apparatus (Bio-Rad Laboratories, CA, USA). Purified proteins from peaks II, II-1, II-2 and pre-immune rabbit serum (BioLegend, USA) were used in the spermatophore wall digestion experiment. Two groups containing purified proteins from peak II-2 (0.5 ml) were mixed with 20 \( \mu \text{l} \) spermatophore suspension (approximately 45 spermatophores). A cathA inhibitor (40 \( \mu \text{g/ml} \), Millipore, USA) was added to one group according to Beck and Lowy (1982). The other group served as a negative control that contained an equivalent volume of Ca2+-FAW. Each group was incubated at 37 \( ^\circ \text{C} \) for 0, 10, 20 and 30 min, and the number of intact spermatophores was counted under a light microscope. Both groups were tested in triplicate.

2.11. ELISA activity assay for cathA

Based on the mass spectrum analysis, cathA activity was measured by an enzyme-linked immunosorbent assay (ELISA) using a Crab cathA (Cath-A) ELISA kit (TSZELISA, USA). All processes were performed as suggested by the manufacturer. The reaction was initiated by adding 50 \( \mu \text{l} \) of appropriately diluted crab Cath-A enzyme and sample dilution to the MicroELISA strip-plate coated with purified crab Cath-A-antibody. After incubation at 37 \( ^\circ \text{C} \) for 30 min, the 96-well plates were washed five times with PBS and 0.5% Tween-20 using a manual washer. Subsequently, 50 \( \mu \text{l} \) HRP-conjugate reagent was added and the plates were incubated at 37 \( ^\circ \text{C} \) for 30 min. Plates were washed to block non-specific binding and Chromogen TMB was added. After incubating under darkness for 10 min at 37 \( ^\circ \text{C} \), 50 \( \mu \text{l} \) of the stopping solution (2 M \( \text{H}_2\text{SO}_4 \)) was added to terminate the reaction (blue to yellow color change). The absorbance of cathA was measured at 450 nm using a Cary 50 UV-visible spectrophotometer (Varian, USA). All enzyme activity assays were performed in triplicates, with variation between duplicate samples always less than 5%, and mean values were used for analysis. One unit for enzymatic concentration activity of cathA was defined as the activity per liter of enzyme.

2.12. CathA inhibitor affected spermatophore wall digestion experiment

In order to confirm whether cathA was the main enzyme involved in digesting spermatophore walls, a cathA inhibitor was used in the spermatophore wall digestion experiment. Two groups containing purified proteins from peak II-2 (0.5 ml) were mixed with 20 \( \mu \text{l} \) spermatophore suspension (approximately 45 spermatophores). A cathA inhibitor (40 \( \mu \text{g/ml} \), Millipore, USA) was added to one group according to Beck and Lowy (1982). The other group served as a negative control that contained an equivalent volume of Ca2+-FAW. Each group was incubated at 37 \( ^\circ \text{C} \) for 0, 10, 20 and 30 min, and the number of intact spermatophores was counted under a light microscope. Both groups were tested in triplicate.

2.13. Statistical analysis

Statistical analysis was performed using SPSS software (Ver. 11.0). The data are represented as mean \pm standard error (SE). Statistical significance was determined by one-way ANOVA and post-hoc Duncan’s multiple range tests. Significance was set at \( P < 0.05 \).

3. Results

3.1. Purification of ASG active protein in spermatophore digestion

The ASG proteins were purified to homogeneity through ammonium sulfate fractionation, Sephadex G-25 S, anion-exchange chromatography using a DEAE-Sephacel column and Sephacryl S-200 gel-filtration followed by ammonium sulfate fractionation. In the anion-exchange chromatography, four peaks (I–IV) were detected and corresponding data was collected (Fig. 1A). The active fractions in spermatophore digestion, eluted in peak II, were then applied to the gel filtration column Sephacryl S-200 for further purification; two peaks (II-1 and II-2) were obtained (Fig. 1B). As a result, protein concentrations of different purification stages were determined (Table 1). The results of the SDS–PAGE analysis of the protein obtained from DEAE-Sephacel and Sephacryl S-200 gel-filtration are shown in Fig 2A and 2B, respectively. The protein from
peak II-2 presented as a single band, corresponding to a molecular mass of approximately 50 kDa (Fig. 2B). Fraction II-2, having the highest activity on spermatophore digestion, was then used for HPLC-ESI-MS/MS analysis.

3.2. Effects of proteins from the different chromatography peaks on spermatophore wall integrity

Purified proteins from different peaks were tested for their function in spermatophore rupture. The results showed that spermatophores (about 40 spermatophores) incubated with other peaks retained their complete shape, such as seen for peak II-1 (Fig. 3A), but that the protein from peak II-2 (1.7 mg) had the ability to rupture the spermatophore wall (Fig. 3B). The spermatophore wall was clearly observed before being mixed with the purified peak II-2 protein (Fig. 3C). As incubation time increased, the spermatophore border could be seen starting to break down (Fig. 3D). The starting time for the release of free sperm by the protein from peak II-2 was 15 min. At 30 min, the walls had completely ruptured, and spermatophores were completely broken down within 45 min (Fig. 3E and F).

3.3. Identification of digestible protein

In order to identify whether trypsin-digested peptides from the purified protein could break down the spermatophore wall, the transcriptome profiling of the testis. ASG databases from *E. sinensis* were searched for profiles that matched our MS/MS spectrum using the SEQUEST program. Based on the HPLC-ESI-MS/MS shotgun analysis, reliable data from protein identification were assembled to five sequences that were consistent with the *E. sinensis* cathA protein sequence (Li et al., 2011). Five peptide fragments containing 95 amino acid residues revealed 100% identity to a cathA sequence ([gi|309380138|) from *E. sinensis* (Fig. 4).

3.4. Western blotting

Western blot analysis revealed that protein bands from peaks II and II-2, with a molecular mass of about 50 kDa, positively reacted with the anti-cathA antibody (Fig. 5). Control serum from the pre-immunized rabbit did not recognize any protein components.

3.5. CathA activity assay

ELISA results on the cathA activity in the ASG of *E. sinensis* fluctuated with the development stages of the ASG (Fig. 6). As the ASG matured, the enzyme activity significantly increased, reaching a peak in the November samples (*P* < 0.05).

3.6. Effects of cathA inhibitor on spermatophore digestion

The impact of a cathA inhibitor on spermatophore digestion is shown in Fig. 7. The number of spermatophores in the cathA inhibitor group was almost indigested. Further, spermatophore numbers in inhibitor group were significantly higher than in the control group after 20 and 30 min when observed under a light microscope (*P* < 0.05).
Fig. 2. SDS–PAGE electrophoresis of purified ASG protein from *E. sinensis*. (A) Protein pattern from DEAE-Sepharose anion-exchange column. Lane M: protein marker; lane ASG: total ASG protein; lane PI: peak I of (A); lane PII: peak II of (A); lane PIII: peak III of (A); lane IV: peak IV of (A). (B) Purified peak II of (A) from Sephacryl S-200 HR column. Lane M: protein marker; lane PII: peak II of (A); lane PII-1: peak II-1 of (B); lane PII-2: peak II-2 of (B).

Fig. 3. Purified protein from peak II-1, II-2 and incubation time on spermatophore digestion. The spermatophore suspension was incubated with peak II-1 (A) and II-2 (B) at 37°C. The spermatophores from peak II-1 (A) still retained their initial shape, but the purified protein from peak II-2 (B) could break down the spermatophore wall. The time to complete spermatophore dissolution was continuously recorded at 0 min (C), 15 min (D), 30 min (E) and 45 min (F). At 15 min, the spermatophore border began to be irregular and the spermatophore wall was completely fractured at 45 min. The spermatophore (dotted arrow) and the sperms (solid arrow) are labeled, respectively. Scale bars: A = 500 μm; B–F = 100 μm.
In the male reproductive system, ASGs are important components that induce many changes to the spermatozoan membrane that influence sperm vitality (Henault et al., 1995; Moura et al., 2007). In addition, proteins secreted by the ASG work as nutritional contributions for newly developed spermatozoa (Gillott, 2003). The purification of secretory proteins and characterization of their activities, including spermatophore digestion, are very important to the knowledge of male reproduction. In *E. sinensis*, the ASG secretory proteins have been shown to participate in digesting the spermatophore wall, which has been confirmed previously (Hou et al., 2010). Therefore, the simple purification method described in the present study might be useful for further studies concerning the role of the ASG in *E. sinensis*.

Here, we employed a DEAE-Sepharose anion-exchange column and a Sephacryl S-200 HR column to purify and obtain the functional protein. All fractions were mixed with a spermatophore suspension to test whether they could yield free spermatozoa. In order to ascertain the target protein, we compared the impact of different peaks on spermatophore digestion. After 30 min, the purified protein from peak II-2 could release sperm from the spermatophore (Fig. 3 E). Within 30 min, the walls were ruptured completely, and spermatophores were totally broken down after 45 min (Fig. 3F). With the help of mass spectrum analysis, the functional protein was identified to be cathA (Fig. 4). Its molecular mass of approximately 50 kDa on SDS–PAGE is similar to that of human cathA (54 kDa) (Bonten et al., 1995; Itoh et al., 2004).

As its name implies, cathA is an important acidic serine carboxypeptidase with deamidase and esterase activities at a neutral pH (Hiraiwa, 1999; Jackman et al., 1990; Nakanishi, 2003). With potential functions in the male reproductive system, it appears to prefer the hydrolysis of aromatic and large hydrophobic amino acid residues at acidic pH (carboxypeptidase activity) and neutral pH (amidase/esterase activity) in the N-terminal amino acids (Bonten et al., 1996; Kawamura et al., 1977; Rottier et al., 1998). As a member of the serine S10 family of proteases, cathA has been identified as the major lysosomal protease in rat kidneys (Shibko et al., 1965). It has also been implicated in the process of autophagy, which occurs after the digestion of type 2a lysosome-associated membrane protein (LAMP2a) (Cuervo et al., 2003). Numerous data indicates that cathA is a multicatalytic enzyme with deamidase and esterase functions in addition to its carboxypeptidase activities (Itoh et al., 1993, 1995; Jackman et al., 1990, 1992). It hydrolyzes a variety of bioactive peptide hormones in vitro, which suggests that extralysosomal cathA plays a role in the regulation of bioactive peptide functions. Using an efficient polyclonal cathA antibody,
Western blot analysis detected the precursor/zymogen form of catA. Our results revealed that one protein band was found in peaks II and II-2 compared to peak II-1, which did not have a band positive to the antibody (Fig. 5). Hence, the presence of catA in peak II-2 further confirmed its connection to spermatoaphore digestion. Other purified proteins did not result in free sperm due to a lack of catA. CatA is one of the most important intracellular lysosome-like hydrolases (Liu et al., 2009). Research has revealed that lysosome-like hydrolases facilitate the degradation of a wide range of macromolecules, such as the catAbolism of Asn-linked glycoproteins to monosaccharides and amino acids (Aronson and Kuranda, 1989) and hybrid organelles produced by heterotypic fusion (Rotter et al., 1998). More than 90% of all proteins are digested by lysosomal proteases with cathepsins as predominant proteases (Hiraiwa, 1999). Previous studies also showed that catA normally exists in an inactive precursor (zymogen) form, which is processed into an active 30 and 20 kDa dimer linked by a disulfide bond (Bonten et al., 1995). Limited by a lack of literature about catA regulation mechanisms in crustaceans, it is difficult to discuss the active fraction of catA. However, the presence of a precursor/zymogen form of catA demonstrates its main function on spermatoaphore digestion. Based on our results and in agreement with Du et al. (1988a), we speculate that catA may induce the slow release of spermatozoa from spermatophores to allow sperm–oocyte interactions and fertilization. ELISA results again confirmed the specificity of catA, as its activity was gradually increased during the purification process. Furthermore, the protein from peak II-2 that could digest the spermatoaphore wall also had the highest catA activity. According to the level of catA in the developmental stages of the ASG, the considerably higher levels in November may be related to the efficient reproductive function in this organ (Fig. 6), since the development of the ASG in E. sinensis lasts from early August until November (Du et al., 1987, 1988a). The pairing time is usually in late autumn (November). Consequently, the activity of catA fluctuated according to the reproductive cycles of E. sinensis. During the mating phase, the ASG secretions along with spermatophores from the seminal vesicle and spermatic fluid enter into the female spermatheca through the ejaculatory duct (Du et al., 1987; Wang et al., 2002). In the spermatheca, the spermatophores are ruptured within a few hours and the spermatozoa are released (Beningier et al., 1988, 1993; Du et al., 1988a,b). This facilitates the interaction of sperm and oocytes to allow fertilization. These phenomena are also indicative of the critical role of catA in breaking down the spermatophore wall in E. sinensis. Indeed, a strong relationship between catA and spermatoaphore digestion has been demonstrated here in vitro.

Furthermore, analysis from a catA inhibitor that affects spermatoaphore wall digestion showed that after a catA inhibitor was added to purified proteins from peak II-2, the digestive function of the purified protein was almost completely abolished. Surprisingly, the spermatophore wall in the group with the added catA inhibitor maintained almost complete integrity and the digestive rate was significantly lower than in the control group. These results demonstrated that catA is the main enzyme involving in spermatoaphore digestion.

5. Conclusion

We successfully purified and identified catA from the ASG of E. sinensis. CatA in peak II-2 (7.58 U/I) had the ability to digest the spermatophore wall, and the appropriate protein concentration was 1.7 mg. The required time for this protein was 30 min, although this time was determined by protein concentration. We hypothesize that, in a natural mating context, environmental parameters and the existence of catA in the ASG may induce the slow release of free sperm. Therefore, this study provides valuable insights into the purification and function of catA in spermatoaphore rupture. CatA has not yet been explored in vertebrates in detail, and further studies will be required to study the spermatoaphore digestion mechanism.

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