Characterization and expression analysis of serpins in the Chinese mitten crab *Eriocheir sinensis*

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**1. Introduction**

Serine protease inhibitors (SPIs) are found in animals, plants and microorganisms, and mainly exist in the granules of plasma or blood cells (Patston 2000). SPIs participate in regulatory processes, including inflammation (Travis and Salvesen 1983), fibrinolysis (Colleen and Lijnen 1991), digestion, fertilization, embryo development (Pak et al., 2004), blood coagulation (Carrell et al., 1991), immune regulation (Ligoyygakis et al., 2003; Levashina et al., 1999) and apoptosis control (Ray et al., 1992). One superfamily of SPIs genes are the serpins, which encode highly homologous proteins of ~350-400 amino acids containing a conserved core structure.

Serpins perform many intracellular and extracellular functions, including roles in fertilization and sperm development. For example, Protein C Inhibitor, a serpin protein secreted into the seminal fluid, can protect sperm, and is depleted during the acrosome reaction (AR) (Elsen et al., 1998). In murine, serpin2 binds to epididymal sperm and may block fertilization by inhibiting sperm capacitation and preventing sperm binding to the egg (Lu et al., 2011). With regards to sperm development, when the epididymspecific secretory serpin known as HongrES1 is co-cultured with rat caudal sperm, there is a significant increase in the percentage of sperm maturation (Hu et al., 2002). Furthermore, knockdown of HongrES1 in vivo reduces fertility in rats, and increases the likelihood of deformed fetuses and pups (Zhou et al., 2008). HongrES1 expression in guinea pig caudal epididymis also reduces gradually during capacitation and completely disappears after the AR (Ni et al., 2009). Therefore, investigating the expression and function of serpins becomes important when studying fertilization and reproduction.

The Chinese Mitten Crab (*Eriocheir sinensis*) is one of the most important cultivated species in Southeast Asia (Ying et al., 2006). With the increase in crab breeding in recent years, understanding the mechanisms behind crab reproductive processes is crucial. Although a diverse range of serpins have been identified in crustaceans, including *Tachypleus tridentatus* (Miura et al., 1994), *Portunus trituberculatus* (Wang et al., 2012a), *Scylla paramamosain* (Chen et al., 2010), *Penaeus monodon* (Somnuk et al., 2012; Homvises et al., 2010), *Exopalaemon carinicauda* (Li et al., 2013) and *Fenneropenaeus chinensis* (Wang et al., 2013a), these studies have mainly focused on gene cloning, tissue distribution and preliminary activity analysis. Moreover, the study of serpins in crustaceans has been limited to understanding their role in the immune response (Wang et al., 2011, 2012a; Zhao et al., 2007).
Indeed, there have been few reports on the activity of serpins during fertilization and reproductive development in E. sinensis to date.

Here we cloned two serpin genes from E. sinensis (Esserpin-2 and Esserpin-3) and investigated the expression of Esserpin-3 in various tissues, and during testis development. In addition, we determined whether Esserpin-3 is involved in the AR by monitoring its expression during an in vitro AR that was induced with egg water.

2. Materials and methods

2.1. Reagents

The reagents used were as follows: TRIzol® (Invitrogen); SMARTer™ RACE cDNA Amplification kit (Clontech); 10X Ex Taq Buffer, dNTP Mixture (2.5 mM), Ex Taq, MiniBEST DNA Fragment Purification kit version 4.0, One Step SYBR® PrimeScript™ RT-PCR kit (Perfect Real Time), SYBR® Premix Ex Taq™ II (Takara); Primers Synthesis (Sanogen Biotech); ZeroBack Fast Ligation kit (TIANGEN); cell lysis buffer for western blots and immunoprecipitation (IP), bicinchoninic acid (BCA) Protein Assay kit, 5× SDS-PAGE sample loading buffer, 0.45 μm PVDF membrane, anti-fade mounting medium (Beyotime); prestained protein ladder (Thermo); cECL western blot kit (CWBio); SP-9001/9002 Histostain-Plus kits, 4,6-diamidino-2-phenylindole (DAPI), FITC-conjugated anti-rabbit IgG (SUNBIO); and Optimal Cutting Temperature (OCT; Sakura).

2.2. Samples and spermatozoa collection

The healthy crabs (100–150 g) were obtained from the Xinan aquatic products market in Shanghai, China from July 2012 to January 2013, and were anesthesia in an ice-bath for 3–5 min until they were lightly anesthetized. The triplicate samples were prepared, gills, hepatopancreas, intestine, muscle, heart, accessory, testis, stomach and brain from three crabs were collected independently. The accessory gland is responsible for the synthesis and secretion of seminal fluid proteins (SFPs). Under transmission and scanning electron microscopy, there are many small and large vesicles in the crab accessory gland, which contained the enzymatic proteins or other activation factors, can effectively digest the spermatophore wall to release free spermatozoa (Hou et al., 2010). Furthermore, it reported that artificial seawater was an excellent solution to preserve E. sinensis sperms in vitro (Chen et al., 2007).

Therefore, a pair of accessory glands in November 2012 (the stage of rapid accessory gland development) was placed in precooled artificial seawater (0.4 M NaCl, 15 mM KCl, 8.6 mM H3BO3, 4.8 mM NaOH and 41 mM MgSO4, pH 7.4) before being punctured with a dissection needle to release the protein. After lumen tissue was discarded, the sample was centrifuged at 1000 g, 4 °C for 10 min and the supernatant (SFPs) wasdigested for subsequent spermophores wall. The spermaphores were obtained from the seminal vesicle and incubated in the supernatant for 15 min at room temperature before being centrifuged at 1000 g, 4 °C for 10 min. Then the supernatant was discarded, and artificial seawater was added. The sample was again centrifuged at 1000 g, 4 °C for 10 min before the sedimented sperm cells were resuspended with artificial seawater.

2.3. Total RNA extraction and cloning of the full-length cDNAs of Esserpin-2 and Esserpin-3

Total RNA was extracted from selected tissues (gills, hepatopancreas, intestine, muscle, heart, accessory gland, stomach and testis), the quality of RNA was verified on 1.5% agarose gels and the concentration was determined by the absorbance at 260 nm (Sambrook and Russell, 2001). Then, 500 ng RNA was used to synthesize cDNA using the SMARTer™ RACE cDNA Amplification kit according to the manufacturer’s instructions.

With the accessory gland transcriptome dataset (He et al., 2013), we found that two gene fragments were homologous to serpins from Litopenaeus vannamei and Pacificastacus leniusculus. These two fragments were selected for further gene cloning. Sequence specific primers were designed to amplify the Open Reading Frame (ORF) (Table1). The amplification reaction was run as follows: 5 cycles of 94 °C for 30 s, 72 °C for 5 min; 5 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min; 28 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 3 min; and 72 °C for 7 min for the final extension step. The purified PCR products were cloned into the pZeroBack/blunt vector, and transformed into Escherichia coli TOP10 competent cells. The potentially positive recombinant clones were identified by colony PCR and picked for sequencing.

2.4. Bioinformatics analysis

VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) was used to remove the vector sequence and splice the remaining sequence. Spliced ORF sequences were analyzed for homology using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast/). The DNAMAN software and the ORF Finder (http://www.ncbi.nlm.nih.gov/OrfPage/OrfReport.htm) were used to analyze the nucleotide sequences and amino acid sequences. Multiple sequence alignments were created using the Clustalw software. The protein domains were predicted by the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). The signal peptide was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The isolectric point and molecular weight were predicted using ExPASy (http://web.expasy.org/protparam/). The phylogenetic tree was constructed using MEGA version 5.0 by the neighbor-joining method based on the serpin amino acid sequence distances and was tested for reliability using 1000 bootstrap replications.

2.5. Real-time PCR analysis of Esserpin-3 mRNA expression

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2.5. Real-time PCR analysis of Esserpin-3 mRNA expression

The mRNA expression of Esserpin-3 in various tissues and during testis development were determined by quantitative and semi-quantitative real-time PCR. The synthesis of first-strand cDNA was carried out according to the protocol in the One Step SYBR® PrimeScript™ RT-PCR kit (Perfect Real Time). Two Esserpin-3 gene-specific primers, Esserpin-3-F and Esserpin-3-R (Table1), were used to amplify a 253 bp product from the cDNA template. The PCR products were sequenced to verify the specificity of the RT-PCR. Chinese mitten crab β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) DNA were used to calibrate the cDNA template as internal controls (Bustin, 2010; Livak and Schmittgen, 2001; Pfaffl et al., 2004). The qRT-PCR was carried out in a total volume of 25 μL, containing 12.5 μL of 2× SYBR® Premix Ex Taq™ II, 1 μL of each PCR primer at 10 μM, 1 μL of diluted cDNA, and 9.5 μL of ddH2O. The PCR program was as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 65 °C for 5 s, and 95 °C for 5 s. To verify the amplification of a single product, melt curve analysis

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used in the experiments.</th>
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<tr>
<td>Primer name</td>
<td>Sequence (5’→3’)</td>
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<tr>
<td>Esserpin-2 5’ primer</td>
<td>AAAGCCCTCTCCATCCTG</td>
</tr>
<tr>
<td>Esserpin-2 3’ primer</td>
<td>ACCAAATGGCACAGC</td>
</tr>
<tr>
<td>Esserpin-3 3’ primer</td>
<td>TCCACATAGGAAAACG</td>
</tr>
<tr>
<td>Esserpin-3 5’ primer</td>
<td>GCCATTCCTCAGGTTCCAT</td>
</tr>
<tr>
<td>qRT-PCR primer</td>
<td>CCAACCTCCAGAAAACAGA</td>
</tr>
<tr>
<td>Esserpin-3-F</td>
<td>GCCCAAGGAGACCAAGC</td>
</tr>
<tr>
<td>Esserpin-3-R</td>
<td>CTCTGCTGTGTACATCATC</td>
</tr>
<tr>
<td>β-Actin-F</td>
<td>GCATCCAGACGACTCTTACA</td>
</tr>
<tr>
<td>β-Actin-R</td>
<td>GTCGAGGACCAAGAAGTG</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>ACGGGACGACGAGCAGT</td>
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<tr>
<td>GAPDH-R</td>
<td>TTATAGCCTACATAGAAG</td>
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<tr>
<td>Sequencing</td>
<td>T7</td>
</tr>
<tr>
<td>SP6</td>
<td>ATTTAGGCTACATAGAAG</td>
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was performed at the end of each PCR reaction. The relative expression level, compared to controls, was determined by the $2^{-}\Delta\Delta Ct$ method (Livak and Schmittgen, 2001). All data were given in terms of relative mRNA expression as means ± standard deviation and were subjected to a one-way analysis of variance (ANOVA) using the SPSS 17.0 program. Differences were considered statistically significant at the level of $P \leq 0.01$.

2.6. Protein extracts and western blot analysis

Total protein extracts of various tissues (accessory gland, testis, gill, intestine, hepatopancreas, stomach, muscle, heart and brain) were prepared according to the cell lysis buffer protocol for western blots and IP. Protein concentration was determined by the BCA Protein Assay kit. Total protein extracts for each sample (10 μg) were separated on 10% (w/v) SDS-PAGE gels and transferred to PVDF membranes (Towbin et al., 1979). Membranes were blocked with 5% (w/v) skim milk in Tris Buffered Saline with Tween 20 (TBST; blocking solution) for 1 h and then incubated with anti-HongrES1 antiserum (1:2000) diluted in NET buffer (0.15 M NaCl, 5 mM EDTA pH 8.0, 50 mM Tris pH 7.5, 0.05% Triton X-100, 0.25 g gelatin) (Ni et al., 2009) overnight at 4 °C. After gentle agitation over three changes of TBST for 10 min, each membrane was immunoreacted with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG), and diluted 1:1000 in NET for 1 h at 37 °C. Immunoreactive bands were revealed using the cECL western blot kit according to the manufacturer’s instructions.

2.7. Immunohistochemical staining of the accessory gland and testis of E. sinensis

Accessory gland and testis tissues were collected and embedded in organ-Tek Optimal Cutting Temperature compound after dehydrated with 30%, 20% and 10% sucrose and fixed with 4% paraformaldehyde. They were then cut into 7 μm sections. The distribution of HongrES1 homologous Esserpin-3 in testis and accessory gland tissue was detected according to the SP-9001/9002 Histostain-Plus kits protocol.

2.8. Immunofluorescence staining of spermatozoa during the egg water induced acrosome reaction in E. sinensis

According to the methods described in Fred et al. (1987) and Zhu et al. (2004), 35–40 g of ovary samples were collected from each female crab that was beginning to ovulate (to give about 300 g total), and the sample was cut into pieces. Shredded ovary samples were soaked in about 200 mL precooling artificial seawater for 24 h at 4 °C and the supernatant ‘egg water’ was reserved. The acrosome reaction was induced with egg water in the dark at room temperature. For indirect immunofluorescence studies of Esserpin-3 in sperm were prepared according to Bi et al. (2009) The reaction was then terminated with 4% paraformaldehyde at 10 min, 20 min, 30 min and 40 min, respectively. Sperm samples were then permeabilized with 1% Triton X-100 in phosphate buffered saline (PBS) for 15 min on ice (this step was omitted from the indirect immunofluorescence experiments with non-permeabilized sperm), followed by blocking with 10% bovine serum albumin (BSA) at room temperature for 1 h. Briefly, after incubated with primary anti-HongrES1 antiserum (at 1:500 dilution) overnight at 4 °C, the slides were treated with FITC-conjugated goat anti-rabbit IgG at a dilution of 1:100 for 1 h at room temperature. Finally, the slides were incubated with DAPI (4′,6-diamidino-2-phenylindole) for 5 min without washing, and mounted in ~100 μL of anti-fade mounting medium before microscopic examination.

3. Results and discussion

3.1. Characterization of Esserpin-2 and Esserpin-3 in E. sinensis

Using sequence specific primers (Table 1), two serpin genes (Esserpin-2 and Esserpin-3) were isolated from E. sinensis. Sequence analysis revealed that Esserpin-2 has a 1383 bp open reading frame (ORF) (GenBank accession number: KJ576784) and Esserpin-3 has a 1311 bp ORF (GenBank accession number: KJ576785). From the deduced amino acid sequences, Esserpin-2 comprises 460 residues (Fig. 1A), with an estimated molecular weight of 51.3 kDa and an isoelectric point of 4.95; and Esserpin-3 comprises 421 residues (Fig. 1B), with an estimated molecular weight of 46 kDa and an isoelectric point of 7.69. Esserpin-2 and Esserpin-3 also contain putative signal peptides as determined using Signal IP software, which suggests they are both secreted proteins. All serpins contain a serpin signature sequence and an exposed reactive center loop (RCL) hinge region located near the C-terminus that acts as a bait for proteases (Carrell et al., 1991). Both Esserpin-2 and Esserpin-3 contained this highly conserved hinge region (EEGTEAAAAT and EEGTVAAGAT), and a serpin signature sequence (FEANSPFLFLI and FNRPFVFLIVD) near the C-terminus (position 21–38 nt) and a serpin domain (Fig. 1C); while serpin8 contained this highly conserved hinge region (EGTTEAAAAT and EGTVAAGAT), and a serpin signature sequence (FEANSPFLFLI and FNRPFVFLIVD) near the C-terminus (position 21–38 nt) and a serpin domain (Fig. 1C); while a serpin domain was identified in Esserpin-3 (Fig. 1D). As there were significant structural differences between Esserpin-2 and Esserpin-3, this indicates that these two serpins may have experienced evolutionary change and formed different functions.

3.2. Sequence comparison and phylogenetic analysis

Esserpin-3 shared amino acid sequence homology with other reported serpins from crustaceans, including 34% identity with serpin (P. leiusculus), 35% identity with serpin7 (L. vannamei), and 35% identity with serpin7 and serpin8 (P. monodon) (Fig. 2). In general, if a protein sequence shares more than 30% sequence identity with a protein from another species, it indicates that the two genes may be members of the same family or belong to the orthologous genes (Zhang and He,
We also examined the conservation of the P1 residue, which determines the target specificity of the serpin to a protease (Gent et al., 2003; Rawlings et al., 2010). The putative P1 residue of Esserpin-3 was arginine, which is equivalent to the P1 residue of serpin-6 from *Manduca sexta*, serpin3, serpin6, and serpin7 from *P. monodon* and serpin from *F. chinensis*, indicating that Esserpin-3 may have inhibitory activity against bovine plasmin, bovine trypsin, prophenoloxidase activating proteases (PAP), clotting enzyme and human tissue plasminogen activator (Kanost and Jiang, 1997; Miura et al., 1994; Wang, 1996).

Our phylogenetic analysis showed that Esserpin-2 first clustered closely with the crustaceans *L. vannamei* (AGZ91893.1), *F. chinensis* (ABC33916.1), *Marsupenaeus japonicas* (BAI50776.1), *P. monodon* (ADC42879.1), *P. leniusculus* (CA57964.1), and then with the Asian butterfly, *Papilio polytes* (BAM19264.1), finally with the Esserpin-3 (Fig. 3). However, both Esserpin-2 and Esserpin-3 were more distant from *Drosophila melanogaster* (CAB63096.1), which instead clustered with *Xenopus laevis* (Q00387), *Rattus norvegicus* (P05545) and *Mus musculus* (P07759) (Fig. 3). This indicates that there was some differentiation in serpin evolution.
3.3. Esserpin-3 mRNA shows differential spatial and temporal expression in *E. sinensis*

We investigated Esserpin-3 expression using quantitative and semi-quantitative RT-PCR methods and β-actin and GAPDH as an internal control. The transcripts of Esserpin-3 were detected in all the examined tissues, including the gill, hepatopancreas, accessory gland, testis, intestine, stomach, muscle and heart (Fig. 4). The highest expression levels were in the gill, hepatopancreas and intestine, followed by the accessory gland, testis and stomach. The muscle and heart showed lowest amount of Esserpin-3 expression (Fig. 4).

Our results are consistent with previously studies on serpin gene expression in crustaceans, which indicated they have different tissue-specific expression. For example, in *F. chinensis*, serpin genes are highly expressed in the hemocytes and gill, but there is low expression in the hepatopancreas (Liu et al., 2009; Homvises et al., 2010). In *Palaemon carinicauda*, serpin gene expression was the highest in hemocytes, with lower levels of expression in the hepatopancreas and muscle, and minimal expression in the gill (Li et al., 2013). On the other hand, no serpin expression was detected in the hepatopancreas of *Procambarus clarkia* (Liang and Soderhall, 1995). In our study, Esserpin-3 mRNA was detected in all examined tissues, with the highest expression in the gill, hepatopancreas and intestines. Immune-related genes (e.g., Toll, Spätzle and Pelle) are often expressed in the gills of crustacean because the gills are the first line of defense against invaders (Wang et al., 2011, 2012a; Zhao et al., 2007). As Esserpin-3 is highly expressed in the gills, it may also function in host defense. In addition, it has reported that BovSERPINA3-1 and A3-3 (belonging to the Serpin superfamily) were located in muscle cells and are cross-class inhibitors strongly active against trypsin as well as against human initiator and effector caspases 8 and 3 (Gagaoua et al., 2015). Several results have tended to ascertain the onset of apoptosis in postmortem muscle, and the level of proteases inhibitor level is a better predictor of meat tenderness than their target enzyme (Boudida et al., 2014). It may be explained that our muscle tissue is postmortem muscle and initiated apoptosis, so the expression level of Esserpin-3 in muscle is very low to active the caspase pathway. However, the wide distribution of Esserpin-3 transcripts observed suggest that it may have multiple biological functions in the Chinese mitten crab.

In addition, we showed that Esserpin-3 was differentially expressed during the development of the testis; its expression level gradually increased from July to November, before reaching a peak in November and then decreasing. However, Esserpin-3 expression from November to January was significantly higher than the expression from July to October (Fig. 5). This temporal expression of Esserpin-3 in the testis indicates that it may be important for sperm development or maturation, similar to the previously reported role of HongrES1 in rats and guinea pigs (Hu et al., 2002).

3.4. Distribution of the Esserpin-3 protein in *E. sinensis*

We used western blotting to study the tissue distribution of the Esserpin-3 protein in *E. sinensis* (i.e., in the gill, hepatopancreas, accessory gland, testis, intestine, stomach, muscle and heart). The HongrES1 antibody recognized a protein of ~46 kDa in size in the accessory gland and testis, which is likely to be the Esserpin-3 protein (Fig. 6). As Esserpin-3 mRNA was detected in the hepatopancreas, gill and...
intestines by qRT-PCR, but Esserpin-3 protein was not detected by western blotting in these tissues, it is likely that Esserpin-3 is under post-transcriptional regulation.

We determined the cell types and subcellular localization of Esserpin-3 protein among the male reproductive tissues using immunohistochemical staining. We showed that the Esserpin-3 protein was localized to the tube wall and lumen of the accessory gland (Fig. 7). The Esserpin-3 protein was also identified in the cytoplasm and cell membrane of spermatogonia, spermatocytes and spermatids, with a significantly higher expression level in spermatocytes than in spermatogonia (Fig. 7).

Recent research suggests that protein secreted from the accessory gland are important for providing nutritional support for sperm, and promoting sperm activity in the female reproduction tract (Elzanaty et al., 2002). For example, in Drosophila, cells of the accessory gland secrete several products (including proteins, carbohydrates, and lipids) into the gland lumen, and these products eventually become part of the seminal fluid transferred to the female uterus during mating (Chen, 1984; Monsma and Wolfner, 1988). Furthermore, proteins from the accessory glands in Holstein cattle participate in key spermatic events, such as motility, capacitation, the AR, sperm–oocyte interactions and protection (Moura et al., 2007). These secreted proteins have also been shown to digest the spermatophore wall, thereby releasing sperm (Hou et al., 2010). Similarly, we previously demonstrated that a protein secreted from the accessory gland in E. sinensis (cathepsin A) aids digestion of the spermatophore wall (Wang et al., 2013b). Therefore, as Esserpin-3 was detected at high levels in the accessory gland of E. sinensis, we further researched whether this protein involved in subsequent spermatic events, including sperm capacitation and release.

3.5. Temporal expression of Esserpin-3 protein in response to an egg water induced acrosome reaction in E. sinensis spermatozoa

Sperm lack intrinsic fertilizing capacity and will only fuse with an oocyte after the AR has occurred. Under normal conditions, the sperm AR take place in the female genital tract and is induced by cumulus cells and the zona pellucida. However, we mimicked the AR process in vitro by using egg water to induce the reaction (Toyoda and Chang, 1974). We used immunofluorescence to detect the expression of Esserpin-3 during the egg water induced AR. Esserpin-3 was detected at a high level in E. sinensis spermatozoa (Fig. 8A, B); however, as the AR proceeded, the level of Esserpin-3 gradually decreased (Fig. 8C–H), and could not be detected at 40 min (Fig. 8I, J). Our results are similar to those observed with HongrES1 and Protein C Inhibitor; the expression of these two serpins were shown to be reduced during the AR (Elisen et al., 1998; Ni et al., 2009). Therefore, Esserpin-3 may control the AR process in E. sinensis in a manner similar to HongrES1 and Protein C Inhibitor as a decapacitation factor.

**Fig. 7.** Immunolocalization of Esserpin-3 in male reproductive tissues. Tissue slices from (A) the accessory gland and (B) the testis incubated with anti-HongrES1 antiserum. The brown area is the positive signal, while the blue area is the nucleus. The seminiferous tubule (SFT) is boxed. SG: spermatogonium, SC: spermatocytes, ST: spermatid.

**Fig. 8.** Immunofluorescent staining of the temporal expression of Esserpin-3 protein in spermatozoa after acrosome reaction induction with egg water. (A, B) Sperm digested by accessory gland (AG) secreted proteins were incubated by egg water. The reaction was terminated by 4% paraformaldehyde at (C, D) 10 min, (E, F) 20 min, (G, H) 30 min, and (I, J) 40 min. Slides were firstly incubated with anti-HongrES1 antiserum, then were treated with FITC-conjugated goat anti-rabbit IgG, and were finally incubated with DAPI. Bar: 0.1 mm.
4. Conclusion

In summary, we cloned two serpin genes from *E. sinensis* and explored the potential reproductive function of Esserpin-3. We provide the first evidence that Esserpin-3 is an intrinsic serpin protein that may be involved in the regulation of sperm maturation and the AR process. In future, investigating serpins and other protease inhibitors in crabs may increase our knowledge of the AR, and help us to develop new management strategies for in vitro sperm culturing.

Acknowledgments

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References


