

Sperm Proteases and Extracellular Ubiquitin–Proteasome System Involved in Fertilization of Ascidians and Sea Urchins

Hitoshi Sawada, Masako Mino, and Mari Akasaka

Abstract

Ascidians (primitive chordates) are hermaphroditic animals that release spermatozoa and eggs almost simultaneously, but some species, including *Halocynthia roretzi*, show strict self-sterility. In *H. roretzi*, a 70-kDa vitelline coat (VC) protein consisting of 12 EGF-like repeats, named HrVC70, appears to be a promising candidate for the self/nonself-recognition (or allorecognition) system during gamete interaction. After spermatozoon recognizes the VC as nonself, sperm 700-kDa extracellular ubiquitin-conjugating enzyme complex appears to ubiquitinate Lys234 of HrVC70, and the ubiquitinated HrVC70 is degraded by the sperm 26S proteasome that is located on the sperm head surface. This novel ubiquitin–proteasome system enables spermatozoa to penetrate through the VC. Sperm trypsin-like proteases, acrosin and spermosin, also participate in fertilization, probably as sperm-side ‘movable’ binding proteins to the VC.

Keywords

Ubiquitin • Proteasome • Sperm • Fertilization • Extracellular • Ascidian

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Introduction

In order to accomplish successful fertilization, it is essential for spermatozoa to specifically bind to and penetrate through the proteinaceous egg coat, referred to as the vitelline coat (VC) in marine invertebrates and the zona pellucida (ZP) in mammals [1–4]. Species-specific recognition and adhesion between spermatozoa and the VC of eggs seem to be critical for marine invertebrates. Self-sterility or self-incompatibility is also beneficial for hermaphroditic organisms, including ascidians, to avoid self-fertilization. Most of these recognition processes are carried out in the interaction between spermatozoa and the VC of eggs, since VC-free eggs are self-fertile and occasionally cross-fertilizable between different species.

In mammals, an acrosomal trypsin-like protease, named acrosin [EC 3.4.21.10], had long been believed to be a lytic agent, lysin, which makes a small hole for sperm passage through the ZP of the oocyte [5–7]. However, since mouse spermatozoa lacking the *acrosin* gene can penetrate through the ZP [8], it is currently believed that acrosin is not essential for the penetration of spermatozoa through the ZP, and that it is involved in the dispersal of acrosomal contents during acrosome reaction [9] and in the secondary binding of spermatozoa to the ZP [10, 11]. However, the physiological substrates of sperm trypsin-like proteases are still debated.

Ascidians (tunicates; primitive chordates) are marine invertebrates occupying a phylogenetic position between vertebrates and ‘true’ invertebrates. Generally, ascidians are hermaphrodites, most of which release spermatozoa and eggs almost simultaneously, but many species show self-sterility or preference for nonself-fertilization rather than self-fertilization. Since VC-free eggs are self-fertile, it is thought that allorecognition takes place in the process of interaction between spermatozoa and the VC of eggs. Therefore, sperm lysin appears to be activated or exposed to the VC after spermatozoon recognizes the VC as nonself.

Ascidians are useful animals for fertilization studies, since fertilization experiments can be easily carried out and also since large quantities of gametes are easily obtained by controlling the seawater temperature and light conditions from the aquacultured species *Halocynthia roretzi* [4]. By using this species, it has been elucidated that the sperm ubiquitin–proteasome system (UPS) plays a key role in the penetration of spermatozoa through the VC, most probably as a lysin. In the present review, we summarize the sperm proteases including the novel extracellular UPS, which are involved in ascidian fertilization, from the viewpoint of posttranslational modification of gamete proteins.

Roles of Sperm Proteases in Fertilization

Structures and Functions of Sperm Trypsin-Like Proteases

Hoshi et al. reported that of various protease inhibitors, trypsin inhibitors such as leupeptin and soybean trypsin inhibitor (proteinaceous inhibitor) and chymotrypsin inhibitors such as chymostatin potentially inhibited the fertilization of intact eggs of

H. roretzi in a concentration-dependent manner [12]. The strong inhibition of fertilization by leupeptin and chymostatin was markedly reduced in the case of VC-free eggs, suggesting that sperm trypsin-like and chymotrypsin-like proteases are involved in the process of sperm binding to and penetrating through the VC [12]. Sawada and his colleagues examined the effects of various fluorogenic peptide substrates on fertilization and found that Boc-Val-Pro-Arg-MCA and Suc-Leu-Leu-Val-Tyr-MCA were the strongest inhibitors of fertilization among trypsin substrates and chymotrypsin substrates, respectively [13, 14]. Two trypsin-like proteases called acrosin and spermosin were then purified from *H. roretzi* sperm using the above trypsin substrate [15]. *H. roretzi* acrosin (HrAcrosin) showed a relatively broad substrate specificity toward peptidyl-Arg-MCAs, but *H. roretzi* spermosin (HrSpermosin) showed a narrow substrate specificity. Boc-Val-Pro-Arg-MCA appears to be a specific substrate among peptidyl-MCA substrates for spermosin. It was suggested that both of these proteases participate in fertilization by comparing the effects of various leupeptin analogs (peptidyl-argininal) on fertilization and enzymatic activities [16, 17], and also by examining the inhibitory effect of anti-spermosin antibody on fertilization [18]. However, the purified enzymes hardly degraded the VC proteins, which are insoluble proteins under physiological conditions (unpublished data). In connection with this, it should be noted that it is still debatable whether mammalian acrosin has the ability to digest the ZP from the same species (see review [3]).

From the structural bases, biological functions of ascidian sperm trypsin-like proteases have been proposed. Both HrProacrosin (precursor of HrAcrosin) and HrSpermosin possess several candidate regions for protein–protein interaction, *i.e.*, two CUB domains in the C-terminus of HrProacrosin and a Pro-rich region in the N-terminus of the light chain of HrSpermosin. Two VC proteins (25-kDa and 30-kDa VC proteins) were identified as binding proteins to these proteases: The 25-kDa VC protein was adsorbed to CUB1-peptide-immobilized agarose beads and HrSpermosin Pro-rich-region-immobilized agarose, while the 30-kDa VC protein was capable of binding to CUB1-peptide-immobilized agarose and GST-CUB1 recombinant protein [19–21]. By cDNA sequencing, it was revealed that the 25-kDa and 30-kDa proteins correspond to the C-terminal region of high-molecular-mass vitellogenin, which belongs to a family of lipid transfer proteins [21, 22]. The 25-kDa protein, which corresponds to a von Willebrand factor D domain, was connected to the N-terminal side of the 30-kDa protein, which corresponds to a ‘C-terminal coding region’ of vitellogenin [23]. Since the hepatopancreas is a major organ expressing vitellogenin, we proposed that vitellogenin is expressed in the hepatopancreas and transferred to the oocytes via bloodstream, during which its C-terminal region might be cut off by a protease(s) and attached to the VC, which in turn may play a role in gamete interactions [21]. Two mRNA species weakly expressed in the ovary, which correspond to the C-terminal regions of vitellogenin called vitellogenin S1 and S2, were recently isolated and sequenced [22]. Vitellogenin S1 and S2 appear to be expressed in oocytes and probably also in test cells, and located at the boundary of the oocyte plasma membrane and test cells. During oocyte maturation, these proteins appear to be released to the perivitelline

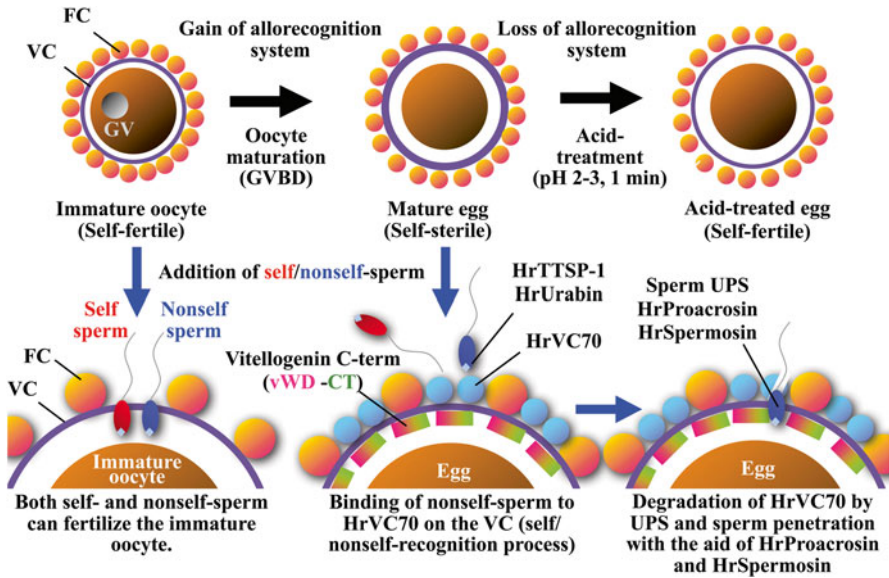


Fig. 1.1 Working hypothesis for the roles of sperm proteases as a lysin and allerecognition system in the ascidian *Halocynthia roretzi* (Modified from [48]). In the ascidian *H. roretzi*, immature oocytes (*upper left*) are self-fertile, but mature eggs (*upper middle*) are strictly self-sterile. When mature eggs are treated with acidic seawater (pH 2–3) for 1 min, self-sterile eggs become self-fertile. This appears to be because HrVC70 consisting of 12 EGF-like repeats, which is an allorecognizable sperm receptor, is attached to the vitelline coat (VC) during oocyte maturation and detached from the VC when treated with 1–10 mM HCl (~pH 2–3). If nonself-spermatozoa recognize the VC as nonself, the sperm-side novel extracellular ubiquitin–proteasome system (UPS) must be activated or exposed to the sperm cell surface, which enables spermatozoa to penetrate through the VC. In this process, sperm trypsin-like proteases HrProacrosin and HrSpermosin may support the movable sperm binding to the VC (for details, see text)

space and eventually attach to the VC from inside. Since these proteins are capable of interacting with sperm proteases HrProacrosin and HrSpermosin, these proteins may participate as scaffold proteins to assist binding and movement of spermatozoa during sperm passage through the VC, although it is presently unclear whether these proteins are derived from oocytes or the hepatopancreas.

In connection with this, we have a working hypothesis that spermatozoa bind to the C-terminal fragments of vitellogenin located on the VC, this process being mediated by the sperm-side HrProacrosin CUB domain and HrSpermosin Pro-rich region, and then sperm proteases may degrade these VC proteins or process the precursor regions, enabling spermatozoa to detach and penetrate the VC. These sequential actions may explain the phenomena of sperm binding to and penetrating through the VC (see Fig. 1.1).

Extracellular Ubiquitin–Proteasome System Functions as a Vitelline-Coat-Lysin in Ascidians

As described above, since the purified preparations of ascidian sperm trypsin-like proteases were unable to degrade the VC, an attempt was made to purify a chymotrypsin-like protease from *H. roretzi* spermatozoa using Suc-Leu-Leu-Val-Tyr-MCA as a substrate. The main proteases that hydrolyze this synthetic substrate were identified as the 20S and 26S (or 26S-like) proteasomes [24–26].

The 26S proteasome is one of the most important intracellular protein-degradation machineries in eukaryotic cells [27, 28]. In this pathway, the intracellular short-lived and aberrant proteins are tagged with ubiquitin by sequential actions of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-ligase E3, and then degraded by the 26S proteasome in an ATP-dependent manner [27–29]. The 26S proteasome is made up of the 20S proteasome, a barrel-shaped protease complex consisting of four stacked heptameric rings, $\alpha7\beta7\beta7\alpha7$, and the 19S regulatory particle (19S RP)/PA700, consisting of 19 subunits, including 6 ATPase subunits and a Ub-recognizing subunit S5a, which caps one or both sides of the cavities of the 20S proteasome [27, 28]. The 20S proteasome has three protease activities, *i.e.*, caspase-like ($\beta1$), trypsin-like ($\beta2$) and chymotrypsin-like ($\beta5$) activities [28].

The 26S proteasome-containing fraction partially purified from activated *H. roretzi* spermatozoa showed a weak VC-degrading activity [24]. It was also found that a 70-kDa main VC component, HrVC70, is degraded by the purified sperm 26S proteasome in the presence of ATP and ubiquitin [25, 26]. HrVC70 consists of 12 EGF-like repeats [25] and appears to be specifically expressed in oocytes [30] within the gonad as a 120-kDa precursor protein HrVC120, which contains a single truncated EGF-like domain and one ZP domain in its C-terminal region [25]. There are several lines of evidence showing the participation of extracellular UPS in ascidian fertilization. First of all, *H. roretzi* fertilization was inhibited by proteasome inhibitors such as MG115 and MG132 and also by an anti-proteasome antibody and the anti-multi-ubiquitin chain-specific monoclonal antibody FK2 [25, 26]. Secondly, Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing proteasome activity, which was specifically inhibited by MG115, was detected in the sperm head region under a fluorescence microscope when activated by alkaline seawater [26]. Thirdly, sperm proteasomes, as well as HrVC70-ubiquitinating enzyme, ATP and ubiquitin, appear to be partially released from sperm when activated by alkaline seawater (unpublished data) [31]. Fourthly, HrVC70 on the VC appears to be ubiquitinated upon insemination on the basis of Western blotting and immunocytochemistry using the monoclonal antibody FK2 [25, 31]. Although the sorting mechanism of the proteasome to the surface of the sperm head is not known, it is notable that only the sperm proteasome possesses an $\alpha6$ subunit lacking the C-terminal 16 residues. This specific processing may be involved in the functions and localization of the sperm proteasome [32].

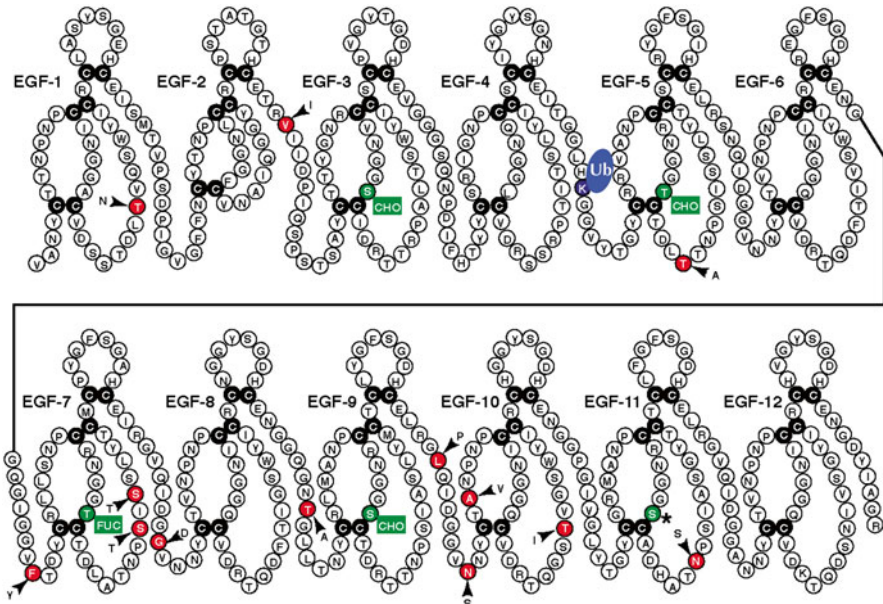


Fig. 1.2 Schematic drawing of HrVC70 [33, 45]. HrVC70 comprises 12 epidermal growth factor (EGF)-like repeats, and shows polymorphisms among individuals. The polymorphic regions indicated by *arrowheads* and (*red*) letters are restricted to the region between the third and fourth Cys residues of each EGF domain and EGF-domain connecting regions. Ubiquitination occurs at Lys234, which is catalyzed by a 700-kDa ubiquitin-conjugating enzyme complex released from spermatozoa upon activation. There are five potential *O*-fucosylation sites (*green*), among which Ser450 (*asterisk*) seems unlikely to be fucosylated as revealed by LC/MS/MS analysis (Sawada et al., to be published)

HrVC70-ubiquitinating enzyme was purified from sperm exudate, a fraction released from spermatozoa activated by alkaline seawater, by DEAE-cellulose chromatography, ubiquitin–agarose chromatography, and 10–40 % glycerol density gradient centrifugation [31]. The molecular size of the enzyme was estimated to be approximately 700 kDa by glycerol density gradient centrifugation [31]. The purified enzyme exhibited activity in artificial seawater and required a high concentration (~10 mM) of Ca²⁺ for its activity. These enzymatic features also support our idea that the purified enzyme functions extracellularly in seawater. Furthermore, apyrase, which depletes ATP and inhibits the HrVC70-ubiquitinating activity, inhibited the fertilization when added to the surrounding seawater. These results indicate that a novel extracellular 700-kDa HrVC70-ubiquitinating enzyme complex plays a pivotal role in ubiquitination of HrVC70. There are two Lys residues in HrVC70, Lys234 and Lys636, but only Lys234 was identified as a ubiquitination site, as revealed by a ubiquitin-conjugation assay using several site-directed Lys-to-Arg mutant recombinant proteins of HrVC70 [33] (see Fig. 1.2). Since it is widely believed that only one molecular species of E1 is committed to every ubiquitination reaction, the existence of the extracellular UPS may give us a new insight in the ubiquitin system.

Involvement of Sperm Proteasome in the Acrosome Reaction and Sperm Penetration of the Vitelline Coat in Sea Urchins

It has been shown that sperm chymotrypsin-like protease is involved in sea urchin fertilization, most probably as a VC lysin, by examining the effects of various protease inhibitors on fertilization of intact and VC-impaired eggs [34]. A chymotrypsin-like protease was then purified from sea urchin spermatozoa, and it was proposed that this enzyme is a VC lysin [35]. However, a high concentration (more than 100 μM) of chymostatin was necessary for inhibiting fertilization, whereas the purified enzyme was very susceptible to chymostatin at lower concentrations. Taking into account the participation of ascidian sperm proteasome in fertilization, we examined the effects of various protease inhibitors, including proteasome inhibitors, on sea urchin fertilization [36, 37]. The results showed that the proteasome inhibitors MG132, MG115 and lactacystin had inhibitory effects on fertilization at a concentration of 100 μM , whereas leupeptin or chymostatin showed no or less inhibition at the same concentration. Proteasome substrates also inhibited fertilization: Among the substrates tested, Z-Leu-Leu-Glu-MCA, a substrate for caspase-like activity, showed the strongest inhibitory effect on fertilization. Proteasome activity was detected in the acrosomal content, a fraction released from acrosome by exocytosis, where the proteasome antigen was detected by Western blotting using an anti-proteasome antibody [37]. MG132 showed no apparent inhibition toward the sperm binding to the VC, but it showed significant inhibition toward fertilization of dejellied eggs using acrosome-reacted spermatozoa. Among three catalytic sites, the caspase-like activity of the proteasome appears to be involved in sea urchin fertilization as revealed by comparing the effects of various protease inhibitors on fertilization and three proteasomal proteolytic activities [37].

It has been proposed that the sperm proteasome is involved in the acrosome reaction (AR) in sea urchins [38]. However, the effects of proteasome inhibitors on the AR had not been studied in detail. Therefore, we re-examined the effects of MG132 on the AR, and we found that MG132 inhibited egg-jelly-induced AR but not Ca^{2+} ionophore-induced AR. From these results, it seems likely that the sperm proteasome plays a key role in the AR, particularly in a certain process leading to the increase in intracellular Ca^{2+} concentration. Taken together, the proteasome is involved not only in the process of sperm penetration through the VC as a lysin but also in the AR before increase in intracellular Ca^{2+} concentration in sea urchin spermatozoa.

Sutovsky and his colleagues showed several lines of evidence indicating that the proteasome is located in an acrosome and involved in the penetration of sperm through the ZP as a lysin in mammals [39–41]. Recently, they succeeded in generating transgenic pigs that express a GFP-PSMA1 ($\alpha 6$) subunit of the proteasome [42]. They reported that the fluorescent proteasome was detected in the acrosome of boar spermatozoa [42]. These results unambiguously demonstrate that the proteasome is localized on the acrosome, although the sorting mechanism of the proteasome into an acrosome is an important issue that remains to be solved. In any case, it should

be emphasized that the sperm proteasome plays an important extracellular role in fertilization, as a lysin, and this lysin system might be commonly utilized in deuterostomes.

Allorecognition in Ascidian Fertilization

It is well known that self-sterile ascidian eggs become self-fertile when the eggs are treated with acidic (pH 2–3) seawater for a short period (~1 min) [43, 44]. It is also known that immature or VC-free eggs are self-fertile [43, 44]. These phenomena led us to speculate that a certain allorecognition factor may be attached to the VC during oocyte maturation and that such a putative factor may be detached from the VC or irreversibly denatured by weak acid. To test this possibility, VCs were isolated from immature and mature eggs and subjected to SDS-PAGE. The results clearly showed that HrVC70 is attached to the VC during oocyte maturation. It was also revealed that HrVC70 is easily solubilized from the isolated VC by 1–10 mM HCl and that spermatozoa are capable of binding to HrVC70 immobilized on agarose beads. It is notable that the number of sperm bound to HrVC70 from nonself-eggs was significantly larger than the number of spermatozoa bound to HrVC70 from self-eggs. In addition, HrVC70 isolated from nonself-eggs more efficiently inhibited the fertilization than did that from self-eggs [45]. From these results, together with the fact that HrVC70 shows high polymorphisms among individuals and that even a single amino-acid substitution in EGF-like repeat regions in Notch protein is sufficient to cause Notch-signaling diseases [46], it is thought that HrVC70 is a promising candidate for allorecognition in fertilization of *H. roretzi*. Although it is still unclear whether the amino-acid substitution in HrVC70 is actually responsible for allorecognition during gamete interaction in *H. roretzi*, all of the biochemical data so far obtained support the idea that HrVC70 is a key protein involved in allongenetic recognition.

As sperm-side binding partners of HrVC70, HrTTSP-1 (Type-II transmembrane serine protease) and HrUrabin (unique RAFT-derived binding partner for HrVC70: a GPI-anchored CRISP-family protein) have been identified by yeast two hybrid screening [30] and Far-Western blot analysis, respectively [47]. HrTTSP1 has an estimated molecular mass of 337 kDa and it contains 23 CCP/SCP/Sushi-domains, 3 ricin B domains and 1 CUB domain in its extracellular region. Although HrTTSP-1 contains several putative interesting domains, its precise function is still unknown. In contrast, HrUrabin appears to play a key role in allorecognition since anti-HrUrabin antibody can inhibit fertilization and also allorecognizable sperm binding to HrVC70-agarose beads. However, HrUrabin had little polymorphism among individuals and showed no difference in its binding ability to HrVC70 from self-eggs and nonself-eggs. Therefore, it is currently thought that HrUrabin is unable to directly distinguish self- and nonself-HrVC70 but that it participates in the allorecognition process since the antibody against HrUrabin potently inhibited the allorecognizable sperm binding to HrVC70.

Conclusions and Perspective

Whereas ascidians are hermaphroditic animals, several ascidians, including *H. roretzi*, show strict self-sterility. In *H. roretzi*, after spermatozoon recognizes the VC as nonself, a novel 700-kDa extracellular ubiquitin-conjugating enzyme complex, which ubiquitinates the Lys234 residue of HrVC70 on the VC, must be activated or exposed to the sperm surface, resulting in sperm penetration of the VC. During this sperm penetration process, it is likely that the sperm HrProacrosin C-terminal CUB-domains and HrSpermosin Pro-rich region are responsible for the sperm binding to the 25-kDa and 30-kDa VC proteins, which correspond to the C-terminus of vitellogenin on the VC. After sperm binding to the above VC proteins, these VC proteins themselves or the binding domains of sperm proteases might be hydrolyzed by sperm HrAcrosin and/or HrSpermosin, which allow sperm movement in the process of sperm penetration of the VC (see Fig. 1.1) [48]. Further studies are necessary to evaluate this working hypothesis.

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