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Extracellular ubiquitin system implicated in fertilization of the ascidian, *Halocynthia roretzi:* isolation and characterization

Naoyuki Sakai,[a] Hitoshi Sawada,[a,b,*] and Hideyoshi Yokosawa[a]

[a] *Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*
[b] *Sugashima Marine Biological Laboratory, Graduate School of Science, Nagoya University, Sugashima-cho, Toba 517-0004, Japan*

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Abstract

The ubiquitin-proteasome system is essential for intracellular protein degradation, but there are few studies of this system in the extracellular milieu. Recently, we reported that a 70-kDa sperm receptor, HrVC70, on the vitelline coat is ubiquitinated and then degraded by the sperm proteasome during fertilization of the ascidian, *Halocynthia roretzi*. Here, we investigated the mechanism of extracellular ubiquitination. The HrVC70-ubiquitinating enzyme activity was found to be released from the activated sperm during the fertilization process. This enzyme was purified from an activated sperm exudate, by chromatography on DEAE–cellulose and ubiquitin–agarose columns, and by glycerol density gradient centrifugation. The molecular mass of the enzyme was estimated to be 700 kDa. The purified enzyme requires $CaCl_2$ and MgATP for activity, and is active in seawater. The purified enzyme preparation, but not the crude enzyme preparation, showed narrow substrate specificity to HrVC70. Moreover, ATP and ubiquitin are released from the activated sperm to the surrounding seawater during fertilization. These results indicate that ascidian sperm release a novel extracellular ubiquitinating enzyme system together with ATP and ubiquitin during penetration of the vitelline coat of the egg, which catalyzes the ubiquitination of the HrVC70, an essential component of ascidian fertilization.

Keywords: Fertilization; Ubiquitin; Proteasome; Sperm; Egg; Ascidian

Introduction

Fertilization is an essential process in sexual reproduction. In contrast to the sperm, ascidian eggs are covered with a follicle cell layer and a noncellular proteinaceous vitelline coat. In order to accomplish successful fertilization, it is crucial for the sperm to penetrate through the vitelline coat to reach the egg. Generally, when the sperm attaches to the vitelline coat, it undergoes the acrosome reaction, an exocytosis of the acrosome. By this reaction, a lytic agent called "lysin" is exposed on the sperm head surface, which makes a small hole in the vitelline coat for sperm penetration (McRorie and Williams, 1974; Morton, 1977; Hoshi, 1985).

In mammals, an acrosomal trypsin-like protease acrosin [EC 3.4.21.10] has been proposed to be a lysin toward the zona pellucida (egg coat). However, by using acrosin gene-knockout mice, it was revealed that acrosin is not essential for sperm penetration through the zona pellucida (Baba et al., 1994; Adham et al., 1997). In order to further characterize the lysin system, we selected the ascidian *Halocynthia roretzi* (Urochordata) as an experimental animal, since large quantities of sperm and eggs are obtainable, and fertilization occurs in seawater, making experiments with *H. roretzi* gametes much more convenient than with mammals.

Ascidians occupy a phylogenetic position between vertebrates and invertebrates, and these animals are hermaphrodites releasing sperm and eggs simultaneously during the spawning season. Interestingly, several species, including *H. roretzi*, are strictly self-sterile because of the occurrence of self–nonself recognition system between sperm and the vitelline coat of the egg (Fuke, 1983). Therefore, it is thought that the lysin system is activated at the sperm head, or exposed and partially released to the surface of the sperm

* Corresponding author. Fax: +81-599-34-2456.
E-mail address: hsawada@bio.nagoya-u.ac.jp (H. Sawada).

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head, after the sperm recognizes the vitelline coat of the egg as nonself (Sawada and Yokosawa, 2001; Sawada, 2002). We have previously reported that two trypsin-like proteases, acrosin and spermosin (Hoshi et al., 1981; Sawada et al., 1982, 1984a,b, 1996; Sawada and Someno, 1996; Kodama et al., 2001, 2002), and proteasomes (Sawada et al., 1983, 2002b; Saitoh et al., 1993) of sperm origin play key roles in sperm penetration of the vitelline coat (see reviews: Sawada, 2002; Hoshi et al., 1994). Although the physiological substrates of spermosin and acrosin remain unknown, a 70-kDa sperm receptor HrVC70 on the vitelline coat was found to be a substrate for the sperm proteasome (Sawada et al., 2002a). We also showed that ubiquitination of HrVC70 is necessary for its efficient degradation by the sperm proteasome (Sawada et al., 2002a).

The ubiquitin–proteasome system plays a pivotal role in intracellular protein degradation. It comprises two systems: one, the ubiquitin system, attaches ubiquitin to the substrate protein (Hershko and Ciechanover, 1998; Peters et al., 1998; Pickart, 2001), and the other is the proteasome system that degrades the ubiquitinated substrate protein (Coux et al., 1996; Tanaka, 1998). The ubiquitin system includes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). But, the extracellular ubiquitin system is nontraditional and is only poorly understood (Sawada et al., 2002a; Sutovsky et al., 2001, 2002). We have reported that HrVC70 is ubiquitinated in vitro by rabbit reticulocyte E1/E2/E3, followed by degradation by the sperm 26S proteasome. Furthermore, the extracellular ubiquitination of the vitelline coat takes place in vivo soon after the addition of sperm as demonstrated by immunocytochemistry. Moreover, the antibody raised against ubiquitin conjugates, which does not cross-react with free ubiquitin, is capable of inhibiting fertilization (Sawada et al., 2002a). These results indicate that extracellular ubiquitination of the vitelline coat protein(s) is necessary for ascidian fertilization, in particular for sperm penetration of the vitelline coat of the egg (Sawada et al., 2002a).

It is reported that ascidian sperm undergoes a sperm reaction, which is defined as vigorous sperm movement and mitochondrial translocation and shedding, in response to the sperm binding to the vitelline coat (Lambert and Epel, 1979; Lambert and Koch, 1988). Since the sperm activation designated as sperm reaction is mimicked by the treatment with alkaline seawater (~pH 9.6) (Lambert and Epel, 1979), and also since the materials released by sperm activation (termed sperm exudate) contain enzymes involved in fertilization, it seems to be important to identify the ubiquitinating enzyme from the ascidian sperm exudate.

In this context, we first tried to identify the ubiquitin-conjugating enzyme activity in the sperm and egg homogenates and also in the sperm exudate. Interestingly and surprisingly, the ubiquitin-conjugating activity was detected not only in the sperm homogenate but also in the sperm exudate. Then, we attempted to isolate the HrVC70-ubiquitinating enzyme from sperm exudate. Here, we show that this enzyme system is able to function on the surface of the vitelline coat during fertilization with the aid of extracellular ubiquitin and ATP.

Materials and methods

Animal, collection of gametes, and sperm exudate

The ascidian *H. roretzi* type A and C (Numakunai and Hoshino, 1980) was used in this study. Sperm and eggs were obtained as described previously (Hoshi et al., 1981; Sawada et al., 1982). Intact sperm (about 100 g) was activated by the addition of an equal volume of artificial seawater containing 20 mM EPPS–NaOH (pH 9.6) (alkaline artificial seawater). After incubation at 13°C for 20 min with gentle agitation, the sperm suspension was centrifuged at 10,000*g* for 20 min to obtain the supernatant, which is called the sperm exudate. A sperm suspension treated with regular artificial seawater (pH 8.0) was centrifuged as described above, and the resulting supernatant was used as a control preparation for the sperm exudate.

Isolation of HrVC70

Ascidian eggs were homogenized with five-fold diluted (20%) artificial seawater containing 0.1 mM diisopropylfluorophosphate to combat proteolysis. The homogenate was filtered through nylon mesh (pore size 150 μm), and the vitelline coat on the blotting cloth was washed extensively with 20% artificial seawater. The purity of the isolated vitelline coats was examined by light microscopy. The settled and packed vitelline coats, which had been previously washed with distilled water, were mixed with an equal volume of 5 mM HCl, followed by gentle agitation using a rotator for 20 min at 4°C. By these procedures, HrVC70 was specifically extracted and fractionated in the supernatant by centrifugation at 10,000*g* for 15 min. The supernatant fraction gave a single band with a molecular mass of 70 kDa by SDS-PAGE (Laemmli, 1970).

Determination of ATP- and protein-concentrations

Extracellular ATP concentration was determined by using a kit for luciferin–luciferase assay (Toyo Ink). Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Immunological procedure and preparation of affinity column

Western blotting with anti-ubiquitin antibody (Sigma) was carried out by a previously described method (Towbin et al., 1979). Ubiquitin-immobilized agarose beads were prepared by using an NHS-activated agarose (Affi-Gel 10; Bio-Rad) according to the manufacturer's protocol. The

ligand content was determined to be 9.0 mg ubiquitin/ml agarose beads by measuring the protein concentration of ubiquitin in the washed out fraction according to the method of Lowry et al. (1951).

Assay for ubiquitin-conjugating enzyme activity

Egg white lysozyme dissolved in 50 mM Tris/HCl (pH 7.5) at a concentration of 1.0 mg/ml was heat-denatured at 100°C for 10 min, and immediately cooled on ice. This process was repeated three times to obtain the heat-denatured lysozyme.

Briefly, [^{125}I]ubiquitin was prepared by adding 0.5 mCi [^{125}I]NaI into 0.5 ml of 10 mg/ml ubiquitin (Sigma) in the presence of chloramin T, and was isolated by Sephadex G-25 according to the manufacturer's protocol of Amersham.

The enzyme sample (10 μg in the case of the crude enzyme preparation, or an equal volume in each chromatographic fraction) was incubated at 20°C for 30 min in 25 μl of 50 mM Tris/HCl (pH 8.0) containing 10 μg of the substrate (HrVC70 or heat-denatured lysozyme), 5 μCi [^{125}I]ubiquitin, 5 mM ATP, 10 mM $MgCl_2$, and 1 mM MG115 (proteasome inhibitor). After incubation, the samples were subjected to SDS-PAGE under reducing conditions by using a 12.5% gel (Laemmli, 1970) followed by autoradiography.

Purification procedure of HrVC70-ubiquitinating enzyme

Purification of the HrVC70-ubiquitinating enzyme by ion exchange and affinity chromatography was carried out according to the previously described method (Hershko et al., 1983). Sperm exudate of *H. roretzi* was applied to a DEAE-cellulose (DE-32; Whatman) column (column volume, 50 ml) equilibrated with 50 mM Tris/HCl (pH 7.5). After the column had been washed with the equilibration buffer, the adsorbed materials were eluted stepwise with 0.2, 0.4, 0.6, and 1.0 M KCl. The active fraction which eluted with 0.4 M KCl was mixed with 5 ml of the ubiquitin–agarose beads equilibrated with the above buffer in a disposable plastic column. This suspension was allowed to incubate at 20°C for 30 min in the presence of 5 mM ATP and 20 mM $MgCl_2$ to form a thioester bond between E1/E2 and ubiquitin by gentle agitation. After the reaction, the ubiquitin–agarose beads were washed with 3 volumes of the equilibration buffer and with 1.5 volumes of the same buffer containing 1.0 M KCl. The tightly bound E1/E2 were specifically eluted with 50 mM Tris/HCl (pH 9.0) containing 20 mM dithiothreitol (DTT) to cleave off the thioester bond between E1/E2 and ubiquitin (Hershko et al., 1983). The active fraction in ubiquitin–agarose chromatography was subjected to 10-40% glycerol density gradient centrifugation by 100,000*g* for 24 h in 25 mM Tris/HCl (pH 7.5) containing 2 mM ATP and 1 mM DTT. The molecular size of the enzyme was estimated by glycerol density gradient centrifugation using thyroglobulin (670 kDa), ferritin (470 kDa), and aldolase (170 kDa) as molecular size standards.

Fertilization assay

Fertilization experiments were carried out according to the method described previously (Hoshi et al., 1981; Sawada et al., 1982). Apyrase (Sigma) was dissolved in filtered seawater buffered with 10 mM EPPS (pH 8.0) and then serially diluted with the same buffered seawater. A portion (5 μl) of sperm or egg (100–200 eggs) suspension was mixed with the apyrase solution (90 μl) in a 96-well flat-bottomed multidish. After gentle agitation for 10 min at 13°C, 5 μl of egg (100–200 eggs) or sperm suspension was added into the preincubated sperm or egg suspension, respectively. After gentle mixing, the fertilized eggs were incubated at 13°C. The eggs were examined at 1 h for the expansion of perivitelline space and again at 2 h for the first egg cleavage under a microscope. The fertilization ratio was determined by counting the number of fertilized eggs, which underwent either of these processes.

Results

Evidence for the presence of HrVC70-ubiquitinating activity in sperm exudate

We have previously reported that HrVC70 of the vitelline coat is specifically ubiquitinated during fertilization of *H. roretzi* (Sawada et al., 2002a). In the present study, therefore, we first attempted to identify the HrVC70-ubiquitinating enzyme activity in the sperm and egg homogenates, and also in the supernatant and pellet after centrifugation (500*g*, 15 min). These crude enzyme preparations were incubated in the presence of MgATP, [^{125}I]ubiquitin, and HrVC70 as a substrate, and the ubiquitin conjugates were detected by SDS-PAGE and autoradiography. As shown in Fig. 1A, the ubiquitinating enzyme activity was detected in the sperm homogenate, but not in the egg homogenate. In addition, the activity appears to be fractionated into the membrane or particulate fractions of the sperm homogenate, suggesting that the enzyme is associated with the sperm membrane fraction. In addition, the HrVC70-ubiquitinating enzyme activity was detected in the sperm exudate, a released fraction from activated sperm (see Fig. 1B). These results fulfill a requisite in localization that the enzyme in question is able to play an extracellular role, particularly on the surface of the vitelline coat during fertilization.

Purification of HrVC70-ubiquitinating enzyme from the sperm exudate

In order to characterize the novel extracellular ubiquitin-conjugating enzyme system, we attempted to purify and characterize the enzyme system, which is responsible for

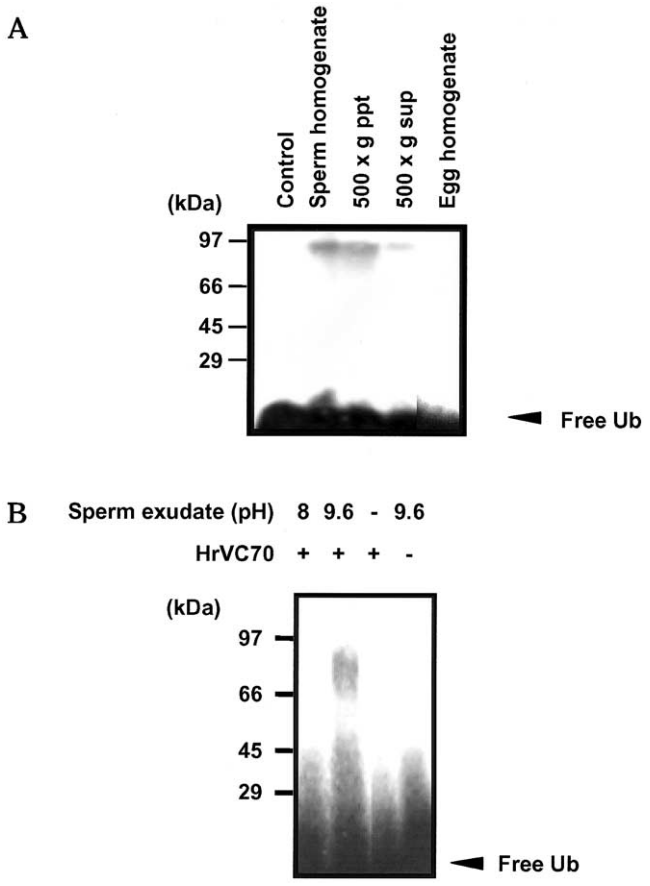

Fig. 1. Existence of HrVC70-ubiquitinating activity. (A) Ubiquitinating activity in the sperm and egg homogenates, and the supernatant and pellet of sperm homogenate. (B) Ubiquitinating activity of sperm exudate. Each fraction (10 μg of protein) was incubated with 10 μg HrVC70 in the presence of 5 mM ATP, 5 μCi [^{125}I]ubiquitin, 10 mM $MgCl_2$, and 1 mM MG115 (proteasome inhibitor). After incubation, the reaction mixtures were subjected to SDS-PAGE, followed by autoradiography.

sperm penetration of the vitelline coat. The HrVC70-ubiquitinating enzyme was purified from the sperm exudate by column chromatography with DEAE–cellulose and ubiquitin–agarose, and by glycerol density gradient centrifugation. The HrVC70-ubiquitinating enzyme activity gave a single peak of activity by glycerol density gradient centrifugation (Fig. 2A). The molecular mass of the enzyme was estimated to be 700 kDa by glycerol density gradient centrifugation (Fig. 2B). The results of SDS-PAGE and silver staining of each fraction showed that the amount of HrVC70-ubiquitinating enzyme is too low to detect the protein bands (data not shown). This suggests that the HrVC70-ubiquitinating enzyme has a very high specific activity and that the enzyme may be made up by several subunits.

Characterization of HrVC70-ubiquitinating enzyme

Since E1 and E2 are able to form a thioester bond with ubiquitin as a reaction intermediate before forming an isopeptide bond between the C-terminal glycine residue of ubiquitin and the lysine residue of substrate proteins (Hershko and Ciechanover, 1998), we investigated whether the purified enzyme complex is capable of forming a thioester bond with [^{125}I]ubiquitin. The purified enzyme preparation was incubated with [^{125}I]ubiquitin and subjected to SDS-PAGE in the presence or absence of 2-mercaptoethanol, which is followed by autoradiography. As shown in Fig. 3A, several bands with molecular masses of 23, 60, 70, and 80 kDa were found to covalently bind to ubiquitin, which is cleaved under reducing conditions. These results suggest that the above-mentioned several bands are candidate E1/E2 enzyme(s). The formation of thioester bonds appears to be essential for the conjugation of ubiquitin to HrVC70, since the ubiquitin conjugates of HrVC70 were not observed in the presence of 20 mM DTT during the ubiquitin-conjugation reaction (Fig. 3B). This coincided with the result that the enzyme adsorbed to ubiquitin–agarose beads in an ATP-dependent fashion was specifically eluted with 20 mM DTT.

As shown in Fig. 4A, both magnesium and ATP are required for ubiquitination of HrVC70. No ubiquitin conjugates were observed by using a nonhydrolyzable ATP ana-

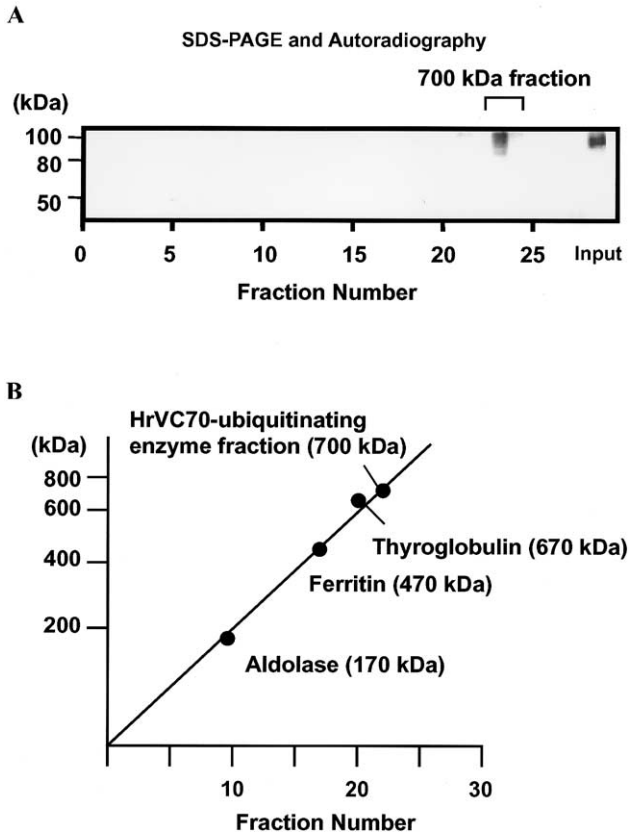

Fig. 2. Purification of HrVC70-ubiquitinating enzyme. (A) Glycerol density gradient centrifugation. Each fraction was incubated with HrVC70 in the presence of 5 mM ATP, 5 μCi [^{125}I]ubiquitin, 10 mM $MgCl_2$, and 1 mM MG115 (proteasome inhibitor). After incubation, the reaction mixture was subjected to SDS-PAGE, followed by autoradiography. (B) Molecular mass estimation by glycerol density gradient centrifugation with thyroglobulin, ferritin, and aldolase, as molecular mass standards.

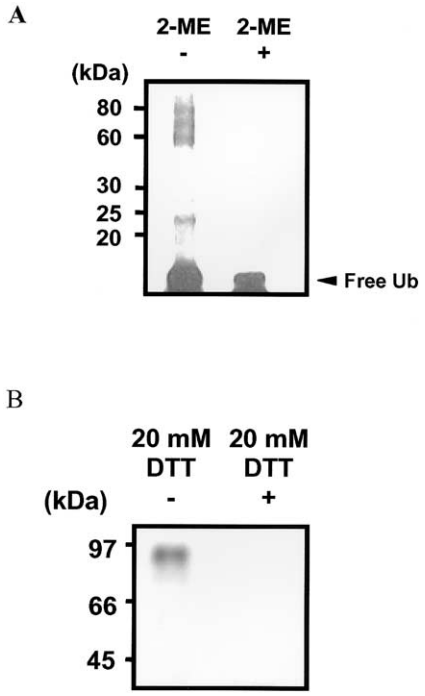

Fig. 3. Occurrence of thioester bond between HrVC70-ubiquitinating enzyme and ubiquitin, and its importance in ubiquitin conjugation. (A) Thioester bond formation between the enzyme and ubiquitin. After the enzyme fraction was incubated with 5 μCi [^{125}I]ubiquitin, 10 mM $MgCl_2$, and 1 mM MG115 (proteasome inhibitor), the reaction mixtures were subjected to SDS-PAGE in the presence and absence of 5% 2-mercaptoethanol (2-ME), followed by autoradiography. (B) Inhibition of ubiquitin conjugation by DTT. The ubiquitin-conjugation reaction to HrVC70 was carried out as described above in the presence (+) and absence (−) of 20 mM DTT. The reaction mixture was subjected to SDS-PAGE and autoradiography.

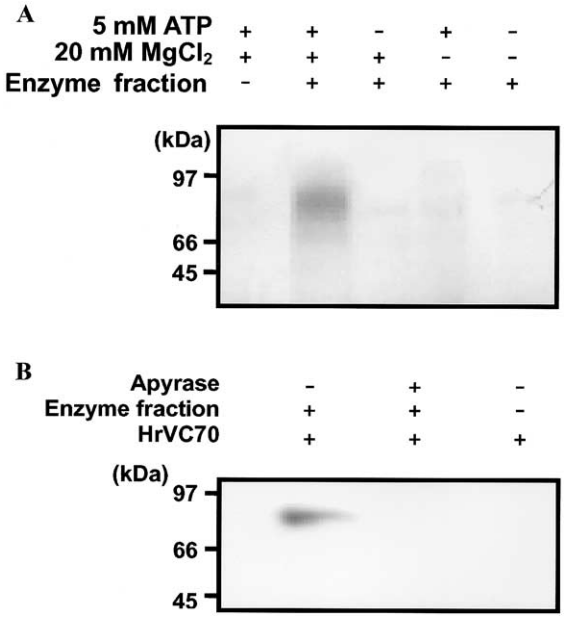

Fig. 4. Requirement of ATP and magnesium ion for the ubiquitinating activity. (A) The ubiquitin-conjugation reaction was carried out as described in Fig. 1 in the presence or absence of ATP and magnesium. (B) Inhibition of ubiquitinating activity by the treatment with apyrase at a concentration of 1 mg/ml. The enzymatic reaction was blocked by the addition of apyrase.

log, AMP-PCP (β,γ-methyleneadenosine 5′-triphosphate), instead of ATP (data not shown). These results indicate that ATP hydrolysis is necessary for ubiquitination of HrVC70. When ATP is depleted by apyrase, ubiquitination of HrVC70 was strongly inhibited (Fig. 4B).

To address the issue of whether the purified enzyme exhibits activity under physiological conditions, optimum pH and cation dependency of the purified enzyme were investigated. The enzyme showed an optimum pH in the activity at about pH 8.0, which is a pH of seawater (Fig. 5A). Effects of Na^+, Ca^{2+}, and K^+ on the activity were examined (Fig. 5B). Ca^{2+} at a concentration of 10 mM, its concentration in seawater, markedly stimulated the conjugating activity. Even at a high concentration (400 mM) of NaCl, the enzyme still has a strong activity. These results indicate that the purified HrVC70-ubiquitinating enzyme is capable of ubiquitinating HrVC70 in seawater during fertilization.

Substrate specificity of the HrVC70-ubiquitinating enzyme

Fig. 6 shows the substrate specificity of the purified and crude preparations of HrVC70-ubiquitinating enzyme from

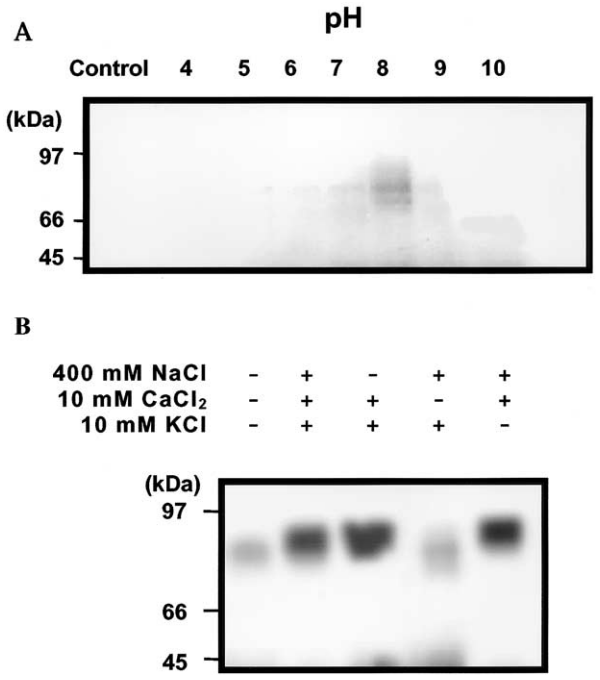

Fig. 5. (A) pH dependency. The enzymatic reaction was performed in the buffer with different pHs as described in Fig. 1 with HrVC70 as a substrate. The reaction mixture was subjected to SDS-PAGE and autoradiography. (B) Ion requirement. The enzymatic reaction was carried out in the presence or absence of cations. The mixture was subjected to SDS-PAGE and autoradiography.

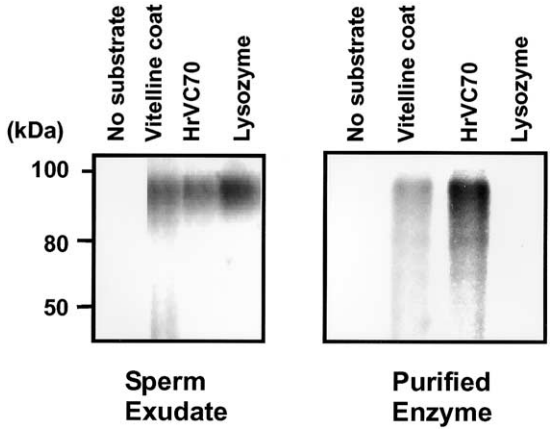

Fig. 6. Substrate specificity of sperm exudate and HrVC70-ubiquitinating enzyme. Sperm exudate and enzyme fraction were incubated with HrVC70 or heat-denatured lysozyme in the presence of [^{125}I]ubiquitin, 1 mM MG115 (proteasome inhibitor), 5 mM ATP, and 10 mM $MgCl_2$. After incubation, the reaction mixtures were subjected to SDS-PAGE, followed by autoradiography.

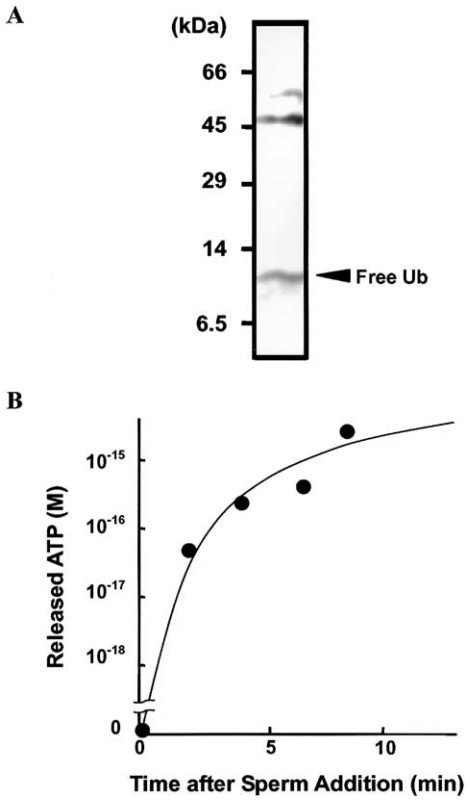

Fig. 7. Existence of ubiquitin and ATP in sperm exudate. (A) Presence of ubiquitin. The sperm exudate was subjected to Western blotting with the anti-ubiquitin polyclonal antibody (Sigma). Arrow indicates the free ubiquitin with a molecular mass of 8.5 kDa. (B) Release of ATP during fertilization. ATP concentration in the supernatant obtained by centrifugation of gametes containing seawater was determined by a kit for luciferin–luciferase assay.

the sperm exudate. Here, heat-denatured lysozyme was used as a control substrate for the ubiquitination reaction, since it is a widely used substrate for ubiquitination in mammals (Hershko and Ciechanover, 1998). Interestingly, we found that the purified HrVC70-ubiquitinating enzyme can ubiquitinate HrVC70 but not heat-denatured lysozyme, although the enzyme preparation in sperm exudate showed broad substrate specificity. These results suggest that the HrVC70-ubiquitinating enzyme in sperm exudate has narrow substrate specificity toward HrVC70, but the other ubiquitin-conjugating enzymes may be contained in the sperm exudate.

Occurrence of ubiquitin and ATP in sperm exudate

Since extracellular ubiquitin and ATP appear to be indispensable for ubiquitination of HrVC70 on the vitelline coat during fertilization, we examined whether ubiquitin and ATP are secreted into the surrounding seawater before penetration of the vitelline coat. As shown in Fig. 7A, free ubiquitin was found to be secreted from the activated sperm as revealed by Western blot analysis. We also found that 10^{-8} M ATP is detected in sperm exudate, and that ATP is gradually secreted to the surrounding seawater during fertilization by measuring the extracellular ATP concentration (Fig. 7B).

We previously reported that the exogenous addition of monoclonal antibody (FK-2) specific for mono- and multiubiquitinated proteins results in inhibition of fertilization and that HrVC70 is multiubiquitinated during fertilization (Sawada et al., 2002a). These results indicate the importance of extracellular ubiquitination of the HrVC70 in ascidian fertilization. In the present study, we investigated the importance of extracellular ATP during fertilization. As shown in Fig. 8, when extracellular ATP is depleted with apyrase, fertilization was strongly inhibited in a concentration-dependent manner. The inhibitory effect due to pretreatment of the sperm was stronger than that due to pretreatment of the egg, suggesting that extracellular ATP is

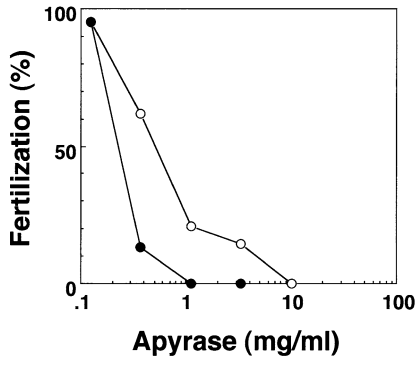

Fig. 8. Effect of the extracellular addition of apyrase on fertilization of *H. roretzi*. After sperm (closed circle) or eggs (open circle) had been incubated with apyrase at a concentration indicated for 10 min, a small volume of egg (100–200 eggs) or sperm suspension was added, respectively. After incubation for 1 and 2 h at 13°C, fertilization ratio was determined as described previously (Hoshi et al., 1981; Sawada et al., 1982).

derived from the sperm rather than the egg. These results unambiguously indicate that extracellular ATP is indispensable for fertilization in *H. roretzi*.

Discussion

In this study, we have demonstrated that the extracellular ubiquitin system to the sperm receptor HrVC70 on the vitelline coat is released from activated sperm during fertilization of the ascidian, *H. roretzi*, and that not only ATP but also ubiquitin is released from activated sperm into the surrounding seawater. In addition, we reported the isolation and characterization of the novel HrVC70-ubiquitinating enzyme complex from the sperm exudate. We selected sperm exudate, rather than sperm homogenate, as a starting material for the purification of the enzyme, since the enzymes in sperm exudate can undoubtedly play an extracellular role in fertilization. In mammals, it is believed that a lytic agent called lysin is exposed on the sperm cell surface or released from sperm during acrosomal exocytosis (McRorie and Williams, 1974; Morton, 1977; Hoshi, 1985). An ascidian acrosome, in contrast, located at the tip of sperm head is too tiny to identify by electron microscopy (Kubo et al., 1978). Although the existence of acrosome in ascidian sperm is currently not doubted, it is still debated whether it is deployed before or after the sperm penetration of the vitelline coat (De Santis et al., 1980; Rosati, 1985; Rosati and De Santis, 1978; Fukumoto and Numakunai, 1993; Fukumoto, 1996). However, it seems probable that the HrVC70-ubiquitinating enzyme system together with ubiquitin and ATP is included in an acrosome of *H. roretzi* sperm and that the enzyme system must be secreted by acrosome reaction from the sperm immediately before or during the process of sperm penetration of the vitelline coat.

Purification of the HrVC70-ubiquitinating enzyme from sperm exudates was performed essentially according to the purification procedures of mammalian ubiquitin-conjugating enzymes (see review: Hershko and Ciechanover, 1998). In mammals, it is well known that substrate specificity in the ubiquitin-conjugation reaction is exhibited by various E2 enzymes (Hershoko and Ciechanover, 1998; Peters et al., 1998; Pickart, 2001). In addition, recent studies revealed that many kinds of E3 complex with HECT or RING domain account for the substrate specificity in the ubiquitination reaction (Hershoko and Ciechanover, 1998; Peters et al., 1998; Pickart, 2001). Since several E3s, including APC or cyclosome, are made up of several subunits, and also since E3 complex can potentially associate with E2 (Pickart, 2001), it is not surprising that the HrVC70-ubiquitinating enzyme system consisting of E1/E2 (and E3) forms a high molecular weight complex. In fact, our present data indicated in Fig. 3 strongly suggest the existence of several subunits that form thioester bonds with ubiquitin. It is most plausible that these bands with molecular masses of 23, 60, 70, and 80 kDa are derived from E1/E2 components in an HrVC70-ubiquitinating enzyme complex (see Fig. 3).

The HrVC70-ubiquitinating enzyme showed an optimum pH around pH 8.0, and it required 10 mM $CaCl_2$ for the activity. In addition, the enzymatic activity was not diminished even at high concentration of NaCl, such as 0.4 M (see Fig. 5). From these results, this enzyme system appears to function efficiently in seawater but not in the sperm cell, since 10 mM Ca^{2+} is required for the enzymatic activity.

In the present study, we showed that ubiquitin is secreted from activated sperm. In the sperm exudate, two additional bands with molecular masses of 45–55 kDa were detected by Western blotting with anti-ubiquitin antibody (Fig. 7A). These cross-reacted bands may be derived from the other ubiquitin-conjugates or ubiquitin-like proteins. In fact, one of the ubiquitin-like proteins designated as SUMO (small ubiquitin-like modifier) and a SUMOylated protein with a molecular mass of 40−45 kDa are known to be present in sperm of the ascidian, *Ciona savignyi* (Inaba, 2000).

In addition to ubiquitin, it was also revealed that ATP is released from ascidian sperm by sperm activation. As indicated in Fig. 7B, the concentrations of ATP released from sperm during fertilization (10^{-15} M) or in the sperm exudate (10^{-8} M) appear to be very low compared with the ATP concentration required for ubiquitination. However, it is reasonable to consider that the concentration of ATP on the surface of the activated sperm head must be high enough for the ubiquitination reaction.

In connection with the importance of extracellular ATP in fertilization, it is intriguing to note that the mammalian sperm acrosome reaction is known to be stimulated via a P2 purinergic receptor by extracellular ATP derived from the genital tract fluid (Foresta et al., 1996; Rossato, 1999; Luria et al., 2002). It is also reported that extracellular ATP of sperm origin induces the increase in permeability of Na^+ in Xenopus egg membrane (Kupitz and Atlas, 1993). The existence and importance of extracellular ubiquitin during fertilization are also reported by others. First, a high concentration of ubiquitin is known to be present in human seminal plasma (Lippert et al., 1993). Second, it is reported that male infertility patients having antibodies to sperm membrane antigens contain anti-ubiquitin antibody (Bohring et al., 2001; Rümke, 1954). Last, the abnormal sperm is ubiquitinated by an extracellular ubiquitination system during passage of epididymis, which is destined to be endocytosed by epithelial cells (Sutovsky et al., 2001, 2002). These reports in conjunction with our findings led us to conclude that the extracellular secretion of ubiquitin and ATP is not an exceptional phenomenon in ascidian fertilization, but it might be a common phenomenon even in mammalian fertilization.

Further studies on the extracellular ubiquitin system, particularly on the identification of E1/E2 (and E3) enzymes, are very intriguing and an important issue remains to be solved, in order to elucidate the new world of the non-traditional ubiquitin system.

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