Sperm proteasomes are responsible for the acrosome reaction and sperm penetration of the vitelline envelope during fertilization of the sea urchin *Pseudocentrotus depressus*

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Abstract

The roles of sperm proteasomes in fertilization were investigated in the sea urchin *Pseudocentrotus depressus*. Two proteasome inhibitors, MG-132 and MG-115, inhibited fertilization at 100 μM, whereas chymostatin and leupeptin showed no inhibition. Among three proteasome substrates, Z-Leu-Leu-Glu-MCA showed the strongest inhibition toward fertilization. MG-132 inhibited the egg-jelly-induced, but not ionomycin-induced, acrosome reaction. In addition, MG-132, but not E-64-d, inhibited fertilization of dejellied eggs by acrosome-reacted sperm. MG-132 showed no significant inhibition toward the binding of reacted sperm to the vitelline layer. Proteasomes were detected by Western blotting in the acrosomal contents, which are partially released upon exocytosis. We also found that the inhibition pattern of the caspase-like activity of the proteasome in the acrosomal contents by chymostatin and proteasome inhibitors coincided well with their inhibitory abilities toward fertilization. Furthermore, the vitelline layer of unfertilized eggs appears to be ubiquitinated as revealed by immunocytochemistry and Western blotting. Extracellular ATP, required for the degradation of ubiquitinated proteins by the proteasome, was also necessary for fertilization. These results indicate that the sperm proteasome plays a key role not only in the acrosome reaction but also in sperm penetration through the vitelline envelope, most probably as a lysin, during sea urchin fertilization.

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Keywords: Proteasome; Sperm; Fertilization; Acrosome reaction; Vitelline layer; Lysin; Sea urchin

Introduction

Fertilization is an essential process for sexual reproduction and is precisely controlled by many gamete proteins. To accomplish fertilization, sperm must penetrate through a proteinaceous egg-coat called the vitelline coat (or vitelline layer or vitelline envelope) in marine invertebrates and the zona pellucida in mammals, which is a formidable barrier to sperm–egg fusion. When sperm enter the jelly coat surrounding the vitelline layer of sea urchin eggs, they undergo the acrosome reaction, an exocytosis of the acrosome. In many invertebrates including sea urchins, this is followed by the formation of an acrosomal process. During this reaction, a lytic agent, lysin, located in the acrosome is exposed and partially released, allowing sperm to penetrate through the vitelline coat.

In mammals, it has long been believed that an acrosomal trypsin-like protease, acrosin, is a zona-lysin (McRorie and Williams, 1974). However, by using mice in which the acrosin gene was knocked out, it was revealed that acrosin is neither essential for sperm penetration of the zona pellucida nor fertilization per se, although a delay was observed during the fertilization process in this animal (Baba et al., 1994). It is currently proposed that acrosin is involved in the dispersal of acrosomal contents during the acrosome reaction (Yamagata...
et al., 1998b) and that the other sperm protease(s) must be zonally in mammals (Yamagata et al., 1998a).

In order to identify the sperm proteases involved in fertilization we have utilized the solitary ascidian Halocynthia roretzi, since large quantities of readily fertilizable gametes are easily obtained from this aquacultured species. We previously reported that two sperm trypsin-like proteases, acrosin and spermomin (Hoshi et al., 1981; Sawada et al., 1984b,c, 1996; Sawada and Someno, 1996) and the sperm ubiquitin–proteasome system (Saitoh et al., 1993; Sakai et al., 2003, 2004; Sawada et al., 1983, 2002a,b) play key roles in the sperm binding to and penetration of the vitelline layer in H. roretzi (see reviews: Sawada, 2002; Sakai et al., 2004; Sawada et al., 2005). In particular, Sawada et al. (2002a,b) demonstrated that the 70 kDa vitelline-coat main component, HrVC70, is ubiquitinated during fertilization, followed by the sperm proteasome-mediated degradation (see reviews: Sawada, 2002; Sakai et al., 2004; Sawada et al., 2005). Very significantly, it was recently reported that sperm proteasomes are also involved in mammalian fertilization (Morales et al., 2003, 2004; Pizarro et al., 2004; Sutovsky et al., 2004). In mammals, sperm proteasomes appear to be responsible not only for the acrosome reaction and sperm binding process to the zona pellucida (Pasten et al., 2005) but also for penetration through the zona pellucida (Morales et al., 2003; Sutovsky et al., 2004). These results led us to speculate that sperm proteasomes may function as lysins also in deuterostomes other than chordates. In order to assess this issue, we here reappraise the identification of lysins in sea urchin fertilization.

In sea urchins, purification and characterization of the proteasome from sperm was reported by Saitoh et al. (1991) and Inaba et al. (1992). It was also reported that the sperm proteasome may be involved in the acrosome reaction in sea urchins (Matsumura and Aketa, 1989, 1991). However, the specific roles of sperm proteasomes in sea urchin fertilization by using several proteasome-specific inhibitors have not yet been studied. In this report, therefore, we systematically investigated the effects of protease inhibitors, including proteasome inhibitors, on the acrosome reaction, sperm binding to and penetration of the vitelline layer, to unambiguously demonstrate the roles of sperm proteasomes in sea urchin fertilization.

Materials and methods

Animal and collection of gametes

The sea urchin, Pseudocentrotus depressus, was obtained near Sugashima Marine Biological Laboratory, Mie prefecture, Japan. Gametes were obtained by intracoelomic injection of 0.55 M KCl. “Dry” sperm were stored on ice until use, while eggs were washed twice with Millipore-filtered seawater (MFSW) before experiments. Dejellied eggs and crude egg jelly were prepared as previously described (Wessel and Vacquier, 2004). Carbohydrate contents in egg-jelly preparations were estimated by the phenol-sulfuric acid method using glucose as a standard (DuBois et al., 1956).

Chemicals

Alexa Fluor 488 phalloidin was obtained from Molecular Probes Inc. (Eugene, OR). MG-132 (Z-Leu-Leu-arginal), MG-115 (Z-Leu-Leu-norvalinal), lactacystin, E-64-d (cell-permeable analog of E-64, a cysteine-protease inhibitor), chymostatin (chymotrypsin inhibitor) and fluorogenic substrates (peptidyl-MCAs) were obtained from Peptide Institute (Osaka, Japan). Leupeptin (acyetyl-Leu-Leu-arginal, a trypsin inhibitor) was kindly provided by Dr. Tetsuya Someno of Nippon Kayaku Ltd. All the other reagents were purchased from Sigma Chemical Co., unless otherwise specified. Inhibitors, except soybean trypsin inhibitor (STI), and fluorogenic substrates were dissolved in DMSO to a final concentration of 10 mM, and stored at –20 ºC until use.

Fertilization assay

“Dry” sperm were suspended in MFSW (about 0.5–1.0 × 10⁸ sperm/ml) and preincubated with respective inhibitors or peptidyl–MCA substrates for 3 min, followed by the addition of 10 μl of egg suspension (about 150–200 eggs) to a final volume of 110 μl. Final concentration of DMSO was adjusted to 1%. To score percentage of fertilization, at least 150 eggs were examined at 5 min after insemination. Fertilization ratio was determined by counting the egg with a fertilization envelope (Hoshi et al., 1979). In experiments where trypsin-inhibitors and -substrates inhibited elevation of the vitelline envelope (Hoshi et al., 1979; Sawada et al., 1984a), the fertilization ratio was determined at 30–60 min after insemination by counting the percentage of cleaved eggs (Hoshi et al., 1979). To examine the effects of proteasome inhibitors on fertilization with acrosome-reacted sperm, sperm were diluted with MFSW containing egg jelly (25 μg carbohydrate/ml) followed by standing for 2 min to induce the acrosome reaction. The reacted sperm (100 μl) was further incubated with various inhibitors for 3 min, followed by the addition of 10 μl of dejellied-egg suspension (about 150–200 eggs) in a 96-well multidish. Sperm concentrations were adjusted to the minimum required for 80–100% fertilization. All the above assays were carried out at 20 ºC.

Assay of acrosome reaction

Assay of the acrosome reaction was carried out as described previously (Biermann et al., 2004; Vacquier and Hirohashi, 2004) with a slight modification. Briefly, dry sperm were diluted with MFSW (1:200), a portion (200 μl) of which was incubated with MG-132 for 5 min. The final concentration of DMSO in each assay was always adjusted to 1%. Then, 10 μl of egg jelly (carbohydrate contents, 5 μg) or ionomycin (final concentration, 10 μM) was added to induce the acrosome reaction. After incubation for 3 min, 200 μl of ice-cold 4% paraformaldehyde in ASW was added and fixed for 1 h at 4 ºC. Fixed sperm were collected by centrifugation (1500×g for 15 min) and resuspended in 500 μl of ASW. To attach the sperm to a cover slip, 50 μl of sperm suspension was placed on a poly-L-lysine-coated cover slip. The sperm were permeabilized with 0.05% (v/v) Triton X-100 and stained with PBS (pH 8.0) containing 0.2 units Alexa Fluor 488 phalloidin, 1 mg/ml BSA and 20 mM glycine, for 2 h under darkness. After washing, sperm with an acrosomal process were scored under a fluorescence microscope using a 100 × Plan Apo objective lens (Nikon) and a filter cube B2A (Nikon).

Sperm binding assay

Binding of sperm to the vitelline layer of the dejellied eggs was carried out as described previously (Kato and Sugiyama, 1978). Briefly, dejellied eggs were fixed with 0.5% glutaraldehyde for 1 h, and then washed repeatedly with MFSW (Hino et al., 1980). The fixed eggs were suspended in MFSW containing egg jelly (25 μg carbohydrates/ml) and 100 μM MG-132, followed by adding the sperm suspension (about 10⁶ sperm/ml). After 2 min, the eggs were washed with MFSW and fixed with 1% glutaraldehyde in MFSW for 30 min at room temperature. Then, the eggs were washed with MFSW three times and sperm nuclei were stained with 0.5 μg/ml 4-diamidino-2-phenylindol (DAPI). The number of sperm bound to a single egg was counted under a fluorescence microscope using a 100 × Plan Apo objective lens (Nikon) and a filter cube B2A (Nikon).

Preparation, Western blotting, and gel filtration of acrosomal contents

“Dry” sperm were washed twice with ASW by two cycles of centrifugation (800×g, 5 min) to carefully remove the possibly contaminating coelomic cells,
and induced to undergo the acrosome reaction with ionomycin at 10 μM. After incubation for 5 min, sperm were precipitated by centrifugation (1500×g, 30 min), and the resulting supernatant was further centrifuged at 17,000×g for 30 min. The supernatant, thus prepared, included the acrosomal contents. The acrosomal contents were subjected to SDS-PAGE in a mini-slab gel (12.5% gel), followed by electronic transfer onto a PVDF membrane. After blocking with TBS containing 3% skimmed milk, the membrane was incubated with anti-proteasome α-subunits monoclonal antibody (Biomol International, PW8195), which reacts with α1, α2, α3, α5, α6 and α7 subunits of the 20S proteasome, for 1 h at room temperature. After washing with TBST, the bands were detected by a horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham) and visualized by the ECL detection kit according to the manufacturer’s protocol. Alternatively, the acrosomal contents were subjected to gel filtration using a Superose 6 column (10×300 mm) equipped with ÄKTA purifier (Amersham Bioscience). Chromatography was carried out using TBS as an elution buffer at a flow rate of 0.5 ml/min, collecting 1 ml fractions.

Assay of enzymatic activity

A portion (150 μl) of the sample solution was mixed with 100 μl of 250 μM fluorogenic peptide substrate dissolved in TBS. After incubation at 37 °C for 12 h, the enzymatic reactions were stopped by adding an equal volume of 2% (w/v) SDS. The activity was determined by measuring the fluorescence intensity at 380 nm excitation and 460 nm emission.

Isolation, SDS-PAGE, and Western blotting of vitelline envelope

Vitelline envelopes were isolated using DTT, according to the method described previously (Correa and Carroll, 1997; Epel et al., 1970). Dejellied eggs (settled volume, 4 ml) were suspended in 10 ml of VE isolation buffer (460 mM NaCl, 50 mM MgCl2, 10 mM KCl, 10 mM CaCl2, 20 mM DTT, 10 mM Tris–HCl (pH 9.0), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin), followed by inverted mixing for 10 min. The egg suspension, thus obtained, was centrifuged (400×g, 3 min), and the resulting supernatant was centrifuged again under the same conditions. The vitelline envelopes in the supernatant were precipitated and repeatedly washed by centrifugation (5000×g, 5 min). The isolated preparation was subjected to SDS-PAGE (7.5% gel), followed by Western blotting using an FK2 monoclonal antibody (1:100 dilution), which specifically reacts to multi- and mono-ubiquitinated proteins but not free ubiquitin (Fujimaro et al., 1994). The reacted bands were detected by an ECL detection kit (Amersham) using an ATTO Light Capture model AE0972.

Immunocytochemistry

Dejellied eggs were fixed with 50 mM MOPS/NaOH (pH 7.0), containing 460 mM NaCl, 50 mM MgCl2, 10 mM KCl, 10 mM CaCl2, and 3% (w/v) paraformaldehyde, for 30 min at room temperature. The fixed eggs were washed five times with MFSW and subjected to blocking with TBST containing 3% (w/v) BSA. After incubation with FK2 monoclonal antibody (5 μg/ml) for 12 h at 4 °C, the eggs were washed five times with PBST, and incubated for 1 h with rhodamine-conjugated anti-mouse IgG antibody (Chemicon) diluted with the buffer used for blocking. Following washing with TBST, the eggs were mounted on a cover slip and observed with a Nikon inverted fluorescent microscope using a filter block G2A (Nikon).

Results

Proteasome inhibitors inhibit sea urchin fertilization

It has been reported that proteasome inhibitors block fertilization in ascidians and mammals (Sawada et al., 1998, 2002b; Sutovsky et al., 2004). Therefore, we first investigated the effects of protease inhibitors, including proteasome inhibitors (MG-132, MG-115 and lactacystin), on sea urchin fertilization using P. depressus. As shown in Table 1, fertilization of P. depressus was strongly inhibited by MG-132, and weakly by MG-115 and lactacystin. These proteasome inhibitors affected fertilization in a concentration-dependent manner (Fig. 1). In contrast to the proteasomal inhibitors, leupeptin, chymostatin, STI and E-64-d showed no appreciable inhibition toward fertilization at 100 μM. It is known that MG-132 is capable of inhibiting the activities of cysteine proteases, including cathepsin K, as well as the proteasomal activity (Votta et al., 1997). However, it is thought that the effects of proteasome inhibitors on fertilization are due to the inhibitory abilities toward the proteasomal activities, since E-64-d, a potent inhibitor for cysteine proteases, showed no significant inhibition toward fertilization (Table 1).

To exclude the possibility that proteasome inhibitors affected egg protease activity, high concentrations of sperm were added to the egg suspension, which had been incubated with 100 μM MG-132. In this experiment, no inhibitory effect on fertilization was observed (data not shown), confirming that it is the sperm proteasome, rather than egg proteasome, that is essential to sea urchin fertilization.

Next, in order to address the issue whether proteasome inhibitors affect the sperm motility, we examined the effect of MG-132 on sperm motility by microscopic observation. No significant difference in the ratio of motile sperm was observed by microscopy in the presence and absence of 100 μM MG-132, which is dissolved in MFSW containing 1% DMSO (data not shown). Our results are not at variance with the results by Hoshi et al. (1979) that not only chymostatin but also all the protease inhibitors tested showed no inhibition toward sperm motility in sea urchins at the concentrations sufficient to inhibit fertilization (Hoshi et al., 1979). These results led us to propose that the inhibitory effects on fertilization of proteasome inhibitors are not due to the effects on sperm motility.

It is well known that there are two complexes in the proteasome, i.e. the 26S and 20S proteasomes, and that the 20S proteasome, a cylindrical particle consisting of four stacked 7-subunit rings (α1–7, β1–7, β′1–7, α′1–7), and a 19S regulatory particle (RP), consisting of about 18 subunits including multi-

<table>
<thead>
<tr>
<th>Inhibitor (100 μM)</th>
<th>Fertilization (% Control)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>MG-132</td>
<td>4.4±1.8</td>
</tr>
<tr>
<td>MG-115</td>
<td>68.5±5.2</td>
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<tr>
<td>Lactacystin</td>
<td>77.0±10.2</td>
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<td>E-64-d</td>
<td>97.4±4.2</td>
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<tr>
<td>Leupeptin</td>
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<tr>
<td>Chymostatin</td>
<td>107±1.6</td>
</tr>
<tr>
<td>STI</td>
<td>104±3.1</td>
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</table>

Sperm suspension (100 μl) was incubated with the respective inhibitor for 3 min, followed by addition of the egg suspension (10 μl) up to a total volume of 110 μl in a multidish. Fertilization ratio was determined by counting the eggs as fertilized that underwent either formation of a fertilization envelope or cleavage. Data represent mean ± S.D. (n=3).
ubiquitin-recognizable subunit S5a, are capable of making up the 26S proteasome in an ATP-dependent manner, which can degrade the ubiquitin-conjugated proteins in an ATP-dependent fashion. It is also known that the 26S and 20S proteasomes possess three peptidase activities: chymotrypsin-like activity (preferred substrate, Suc-Leu-Leu-Val-Tyr-MCA; catalytic site, $\beta_5$-subunit), trypsin-like activity (Boc-Leu-Arg-Arg-MCA; $\beta_2$-subunit), and caspase-like activity (Z-Leu-Leu-Glu-MCA; $\beta_1$-subunit) (Mayer et al., 2004). Therefore, in order to obtain insights into the catalytic centers of the proteasome participating in sea urchin fertilization, we examined the effects on fertilization of three peptide substrates for the proteasome. As shown in Fig. 2, Z-Leu-Leu-Glu-MCA and Boc-Leu-Arg-Arg-MCA inhibited fertilization in a concentration-dependent manner. In contrast, Suc-Leu-Leu-Val-Tyr-MCA showed no appreciable inhibition at 100 $\mu$M. This suggests that caspase-like activity, rather than trypsin-like or chymotrypsin-like activity, of the proteasome plays a critical role in sea urchin fertilization.

**MG-132 inhibits the egg jelly-induced acrosome reaction**

After determining that the proteasome is involved in sea urchin fertilization, we explored which step(s) are blocked by proteasome inhibitors. It was proposed by Matsumura and Aketa (1989, 1991) that the proteasome is involved in the acrosome reaction of sea urchins, since chymostatin and Suc-Leu-Leu-Val-Tyr-MCA inhibit the acrosome reaction, albeit weakly (>$200$ $\mu$M). However, it has not been tested whether proteasome-specific inhibitors are capable of inhibiting the acrosome reaction. We investigated the effect of MG-132 on the acrosome reaction by counting the sperm forming an acrosomal process, which was visualized by using fluorescent phalloidin according to the method described previously (see Fig. 3A) (Biermann et al., 2004; Vacquier and Hirohashi, 2004). As shown in Fig. 3B, the egg-jelly-induced acrosome reaction was inhibited by MG-132.
100 μM MG-132, but the ionomycin (Ca\(^{2+}\) ionophore)-induced acrosome reaction was not inhibited at all (Fig. 3B). Similar results were obtained in different batches of gametes (data not shown). These results suggest that the proteasome participates prior to the increase in intracellular Ca\(^{2+}\) concentration, during the acrosome reaction.

Proteasomal inhibitor also blocks fertilization with acrosome-reacted sperm

In ascidians (Sawada et al., 1998, 2002a,b) and mammals (Sutovsky et al., 2004), it is reported that the proteasome is involved in sperm penetration of the proteinaceous egg-coat. However, such a function of the sperm proteasome has not yet been proposed in sea urchins. In order to assess this issue, we investigated the effect of MG-132 on the fertilization of dejellied eggs using acrosome-reacted sperm, to distinguish the inhibitory effect of MG-132 on the acrosome reaction. Since the fertilization ability of the acrosome-reacted sperm appears to decrease rapidly, dejellied eggs were used in this study instead of intact ones. Soon after inducing the acrosome reaction, sperm were incubated with MG-132, followed by mixing with dejellied eggs. As shown in Fig. 4, the fertilization of dejellied eggs with reacted sperm was inhibited by MG-132 in a concentration-dependent manner. In contrast, E-64-d showed no inhibition at 100 μM. These results indicate that the sperm proteasome also participates in a fertilization process after the acrosome reaction.

Effect of MG-132 on sperm binding to the vitelline layer

We investigated whether the proteasome is involved in sperm binding to the vitelline coat, as reported in the ascidian H. roretzi (Takizawa et al., 1993). In sea urchins, it is currently believed that a sperm receptor on the vitelline envelope is either released or degraded by a trypsin-like protease released from cortical granules upon fertilization (Vacquier and Moy, 1977; Vacquier et al., 1973). To avoid degradation of the sperm receptor, glutaraldehyde-fixed eggs were used in this study. As shown in Fig. 5, MG-132 showed no significant inhibition toward the binding of sperm to the vitelline layer, suggesting that the proteasome is not essential for sperm binding to the vitelline envelope. Taken together, it is most plausible that the sperm proteasome is involved not only in the acrosome reaction, but also in sperm penetration of the vitelline layer, rather than binding to the vitelline coat.

Acrosomal contents have the proteasome antigen and activity

If sperm proteasomes function as lysins, they should be partially secreted from the acrosome during the acrosome reaction. In order to assess this issue, we examined whether proteasomes are detected in acrosomal contents by Western blotting. We also investigated whether the proteasome activity is detected in acrosomal contents using three fluorogenic peptide substrates for the proteasome. As shown in Fig. 6, the proteasome was found to be present in the acrosome-reacted sperm supernatant by Western blotting, using a proteasome-specific monoclonal antibody. In contrast, no cross-reacted band was observed in a control experiment lacking the primary antibody. The acrosomal contents showed peptidase activities toward Suc-Leu-Leu-Val-Tyr-MCA, Boc-Leu-Arg-Arg-MCA and Z-Leu-Leu-Glu-MCA. MG-132 strongly inhibited the chy-
motrypsin-like and caspase-like activities in the acrosomal contents (Fig. 7), while this inhibitor showed a weak inhibition to the trypsin-like activity (Fig. 7). Leupeptin was the strongest inhibitor against Boc-Leu-Arg-Arg-MCA activity among the inhibitors tested (Fig. 7). On the other hand, chymotrypsin-like and caspase-like activities were only slightly inhibited by leupeptin. Chymostatin was a moderate inhibitor toward the chymotrypsin-like and trypsin-like activities, but it showed no appreciable inhibition toward the caspase-like activity even at 100 μM (Fig. 7). E-64-d showed no significant inhibition toward the three peptidase activities (Fig. 7).

Since several proteases could be released from the acrosome in addition to the proteasome, the acrosomal contents were subjected to Superose 6 gel filtration to separate any such proteases. It is thought that the 26S proteasome (about 2500 kDa) and the 20S proteasome (700 kDa) are fractionated into fractions 8 and 12, respectively, under our experimental conditions (for the elution positions of the 26S and 20S proteasomes, see Sawada et al., 1993). As expected, three peptidase activities toward Suc-Leu-Leu-Val-Tyr-MCA, Boc-Leu-Arg-Arg-MCA and Z-Leu-Leu-Glu-MCA, residing on the 26S and 20S proteasome, were eluted in fractions 8 and 12 (see Fig. 8). In addition to the proteasome activities, chymotrypsin-like and trypsin-like activities were detected in fraction 16. These activities with a molecular mass of about 70 kDa are thought to be distinct from the proteasome on the basis of molecular size and enzymatic properties (Fig. 8). In order to exclude the possibility that the 70 kDa chymotrypsin-like protease is also involved in sea urchin fertilization, we compared the effects of MG-132 on the chymotrypsin-like activities in fractions 12 and 16. The chymotrypsin-like activity in the proteasome-containing fraction 12 was strongly inhibited by 100 μM MG-132, while the activity in the 70 kDa protease-containing fraction 16 was scarcely inhibited by MG-132 at the same concentration (Table 2). These results indicate that the protease in acrosomal contents have proteasomal activity. Acrosomal contents were incubated with 100 μM of fluorogenic substrates in the presence or absence of 100 μM inhibitors for 12 h. The activity was determined by measuring fluorescence intensity of AMC. Three kinds of proteasome activity were observed in the acrosomal contents: all the activities were inhibited by MG-132. Similar results were also obtained by using two preparations of acrosomal contents obtained from different individuals.

Fig. 8. Gel filtration of acrosomal contents. Acrosomal contents were fractionated by Superose 6 FPLC. Each fraction was incubated with 100 μM of fluorogenic substrates, Suc-Leu-Leu-Val-Tyr-MCA (for chymotrypsin-like activity), Boc-Leu-Arg-Arg-MCA (for trypsin-like activity), and Z-Leu-Leu-Glu-MCA (for caspase-like activity). The activity was determined by measuring the fluorescence intensity of AMC. The chymotrypsin-like and trypsin-like activities afforded three peaks, while the caspase-like activity gave two peaks. The elution positions of two molecular size markers (porcine thyroglobulin (669 kDa) and bovine serum albumin (66 kDa)) were indicated in the upper panel. Note that fractions 8 and 12 correspond to the elution positions of the 26S and 20S proteasomes, respectively, both of which possess chymotrypsin-like, trypsin-like, and caspase-like activities.
The vitelline envelope of unfertilized eggs is ubiquitinated

It is generally believed that the 26S proteasome degrades ubiquitinated proteins in an ATP-dependent fashion under physiological conditions, although several exceptional cases such as ornithine decarboxylase and CDK inhibitor p21CIP, both of which are degraded by the proteasome in a ubiquitin-independent manner (Mayer et al., 2004), are known. When the sperm proteasome functions as a lysin, it is assumed that the vitelline envelope of the egg may be ubiquitinated before or after sperm binding to the egg vitelline layer. In the ascidian, *H. roretzi*, the vitelline coat of the egg appears to be ubiquitinated by a ubiquitin-conjugating enzyme complex released from sperm during sperm reaction (Sawada et al., 2002a; Sakai et al., 2003). In contrast, it is reported that the zona pellucida of the porcine ovum is ubiquitinated before contact with acrosome-reacted sperm (Sutovsky et al., 2004). To investigate whether the sea urchin vitelline envelope is ubiquitinated, we carried out Western blotting and indirect immunofluorescence microscopy. To avoid possible contamination of cytoplasmic ubiquitinated protein, the purified preparation of the vitelline envelope (Correa and Carroll, 1997; Epel et al., 1970) was used for SDS-PAGE analysis and Western blotting. As shown in Fig. 9A, smear bands with molecular masses more than 50 kDa were detected by the monoclonal antibody FK2, which is specific to the mono- and multi-ubiquitinated proteins but not free ubiquitin (Fujimuro et al., 1994). No reacted band was observed in a control experiment without using the primary antibody (data not shown). Immunocytochemistry of unfertilized eggs was also carried out using the FK2 monoclonal antibody. FK2-reactive proteins were mainly detected on the outer surface of the vitelline layer (Figs. 9B, C). Similar results were obtained by confocal fluorescence microscopy: only the vitelline layer was stained by fluorescence-labeled FK2 antibody (data not shown). These data suggest that the vitelline envelopes of sea urchin eggs are ubiquitinated prior to fertilization.

Extracellular ATP is required for sea urchin fertilization

Extracellular ATP is necessary for fertilization in the ascidian, *H. roretzi* (Sakai et al., 2003). In the case of sea urchins, extracellular ATP may also be required for degradation of the ubiquitinated vitelline-coat proteins by the sperm proteasome. In order to assess this issue, we investigated the effect on fertilization of the addition of apyrase to deplete the extracellular ATP in the seawater surrounding the eggs. As shown in Fig. 10, apyrase inhibited sea urchin fertilization in a dose-dependent manner (Fig. 10), whereas BSA showed no inhibition even at 1.0 mg/ml. Using heat-denatured apyrase drastically diminished its inhibitory ability toward fertilization (data not shown). These results indicate that extracellular ATP is required for fertilization in the sea urchin, as it is in the case of the ascidian *H. roretzi*.

**Table 2**

<table>
<thead>
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<th>Inhibitor</th>
<th>Chymotrypsin-like activity in Superose 6 fractions (% activity)</th>
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<tr>
<td>Fraction 12 (20S proteasome)</td>
<td>Fraction 16 (70 kDa protease)</td>
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Chymotrypsin-like activities in fractions 12 (20S proteasome) and 16 (70 kDa protease), which were separated from the acrosomal contents by Superose 6 gel filtration, were assayed in the presence and absence of 100 μM MG-132 by using Suc-Leu-Leu-Val-Tyr-MCA as a substrate.

**Fig. 9.** The vitelline envelope of unfertilized eggs is ubiquitinated. (A) Vitelline envelopes were isolated from unfertilized eggs and subjected to SDS-PAGE (7.5% gel), followed by Western blotting with an FK2 monoclonal antibody, which reacts to mono- and multi-ubiquitinated proteins but not to free ubiquitin. (B and C) Immunocytochemistry of unfertilized eggs with FK2 monoclonal antibody (B) or control mouse IgG (C).

**Fig. 10.** Extracellular ATP is required for sea urchin fertilization. Sperm was mixed with various concentrations of apyrase (final volume, 100 μl), which deplete the extracellular ATP, followed by addition of a small volume (10 μl) of intact egg suspension. Fertilization ratio was determined as described in Materials and methods. BSA was used as a control. Data represent mean±S.D. in three experiments.
Although we cannot rule out the possibility that extracellular ATP is necessary for several fertilization processes other than proteasome-mediated degradation, the present results support our hypothesis that the ubiquitinated vitelline-coat proteins may be degraded in an ATP-dependent fashion by the sperm 26S proteasome, which is exposed and partially released during acrosome reaction.

Discussion

It has been reported that the proteasome occurs in sea urchin sperm (Saitoh et al., 1991; Inaba et al., 1992) and that the sperm proteasome may be involved in the acrosome reaction (Matsumura and Aketa, 1989, 1991). However, the effects of proteasome-specific inhibitors on several fertilization processes have not been systematically investigated. In the present study, we have demonstrated that the proteasome is involved in sea urchin fertilization at specific steps of the acrosome reaction and penetration through the vitelline envelope by using proteasome-specific inhibitors. We also show that the sperm proteasome is partially released by sea urchin sperm during the acrosome reaction and plays an extracellular role, most probably as a lysin, in the degradation of ubiquitinated vitelline-coat proteins, similarly to ascidians (Sawada et al., 1998, 2002a) and mammals (Sutovsky et al., 2004).

Our present results show that the sperm proteasome plays a crucial role in the fertilization of echinoderms as well as mammals and protochordates. In addition to the inhibitors, synthetic peptide substrates for the proteasome also inhibited fertilization. Z-Leu-Leu-Glu-MCA, a caspase substrate, showed the strongest inhibition. These results led us to propose the idea that a caspase-like catalytic site (β1 subunit) (Mayer et al., 2004) of the 20S proteasome may be critically involved in the degradation of ubiquitinated vitelline-coat proteases. Such an unequal contribution of three catalytic sites of the proteasome in the degradation process has been reported (Tanaka et al., 2000; Kisselev et al., 2006). In connection with this, it is notable that caspase-like activity of the proteasome contained in acrosomal contents was potently inhibited by MG-132 but not by chymostatin or leupeptin at 100 μM (Fig. 7), and that this inhibition pattern coincided well with the inhibition pattern toward fertilization (Table 1). These results also support the idea that caspase-like catalytic center of the proteasome must play a pivotal role in sea urchin fertilization.

It is proposed that a chymotrypsin-like protease is a lysin in sea urchin fertilization, since two chymotrypsin inhibitors (chymostatin and tosyl-phenylalanyl-chloromethane) and p-nitrophenyl-p'-guanidino-benzoate, but not the other protease inhibitors, including leupeptin, inhibited fertilization of the sea urchins Hemicentrotus pulcherrimus and Strongylocentrotus intermedius at concentrations higher than 200 μM (Hoshi et al., 1979). Our data are not at variance with their results, since our preliminary data showed that chymostatin at concentrations higher than 200 μM showed a significant inhibition toward fertilization (data not shown). A 19-kDa chymotrypsin-like protease has been purified from sea urchin sperm (Yamada et al., 1982). It is proposed that this protease may be a vitelline-coat lysin, since the purified enzyme has a vitelline-envelope lytic activity. However, since the activity is potently inhibited by low concentrations (less than 100 μM) of chymostatin and STI, it seems unlikely that such a chymostatin-sensitive protease is indispensable for sperm penetration through the vitelline envelope in sea urchins under physiological conditions.

Our results support the earlier observation that the proteasome may be involved in the acrosome reaction of sea urchin sperm (Matsumura and Aketa, 1989, 1991). In connection with this, it is interesting to note that PKA appears to be involved in the acrosome reaction of sea urchins (Su et al., 2005). Taking into account that the regulatory subunits of PKA are known to be degraded by the proteasome (Hegade et al., 1993), it is reasonable to speculate that the sperm proteasome may play a role in the degradation of the regulatory subunits of PKA resulting in irreversible activation of PKA during the acrosome reaction of sea urchins. In connection with a membrane fusion event, it is reported that the proteasome is involved in yeast vacuole membrane fusion (Kleijnen et al., 2007), although the target for the proteasome remains to be elucidated. Further studies are necessary to clarify the physiological substrate(s) for the proteasome during the acrosome reaction, in order to obtain insights on the signal transduction pathway leading to the acrosome reaction.

It was proposed that STI-sensitive protease is localized on the surface of the acrosomal process of sea urchin sperm (Green and Summers, 1980). Our data also suggest that a ~70 kDa trypsin-like and also chymotrypsin-like proteases are partially released during acrosomal exocytosis (see Fig. 8). However, the occurrence of a certain protease in an acrosome does not necessarily mean that the enzyme in question is involved in fertilization, since not only STI but also chymostatin or leupeptin showed no appreciable inhibition at 100 μM toward fertilization of sea urchins. Although we cannot rule out the possibility that the 70 kDa or other proteases are involved in some steps during fertilization, it should be emphasized that the proteasome, in particular its caspase-like catalytic site, must play a central role in sperm penetration of the vitelline layer. Identification of the ubiquitinated vitelline-coat proteins of sea urchin eggs as well as examination of whether protostomes also utilize sperm proteasomes as lysins is an intriguing issue remaining to be solved.

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References

A thorough literature review on the role of the sperm proteasome during fertilization. The proteasome, a multicatalytic proteinase, is involved in the degradation of regulatory proteins involved in the acrosome reaction and sperm adhesion. The study highlights the importance of chymotrypsin-like proteases in fertilization, with a focus on the role of the sperm proteasome in the regulation of membrane fusion during fertilization. The research also discusses the involvement of ubiquitination and proteolysis in the regulation of sperm proteins.

Key findings include:

1. **Chymotrypsin-like proteases:** These enzymes play a crucial role in the acrosome reaction and sperm adhesion. Inhibitors of chymotrypsin-like proteases have been shown to block these processes.

2. **Ubiquitination and proteasome-mediated degradation:** Ubiquitin plays a key role in the regulation of sperm proteins. The proteasome is involved in the degradation of regulatory proteins involved in the acrosome reaction.

3. **Localization and expression:** The sperm proteasome is localized to the acrosomal region, and its expression is regulated during fertilization.

4. **Function in fertilization:** The sperm proteasome is involved in the degradation of regulatory proteins involved in the acrosome reaction and sperm adhesion. It plays a crucial role in the regulation of membrane fusion during fertilization.

The study concludes that the sperm proteasome is a key player in the regulation of fertilization processes, and its disruption could have significant implications for fertility and fertility-related disorders.
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