

Sperm proteasome degrades egg envelope glycoprotein ZP1 during fertilization of Japanese quail (*Coturnix japonica*)

著者	Sasanami Tomohiro, Sugiura Kenichi, Tokumoto Toshinobu, Yoshizaki Norio, Dohra Hideo, Nishio Shunsuke, Mizushima Shusei, Hiyama Gen, Matsuda Tsukasa
journal or publication title	Reproduction
volume	144
number	4
page range	423-431
year	2012-08-02
出版者	Society for Reproduction and Fertility
権利	(C) 2012 Society for Reproduction and Fertility
URL	http://hdl.handle.net/10297/7053

1 **Title:** Sperm Proteasome Degrades Egg Envelope Glycoprotein ZP1 During
2 Fertilization of Japanese Quail (*Coturnix japonica*).

3
4 **Author's names:** Tomohiro Sasanami¹, Kenichi Sugiura¹, Toshinobu Tokumoto²,
5 Norio Yoshizaki³, Hideo Dohra⁴, Shunsuke Nishio⁶, Shusei Mizushima¹, Gen Hiyama⁵
6 and Tsukasa Matsuda⁶

7
8 **Addresses:** ¹Department of Applied Biological Chemistry, Faculty of Agriculture,
9 Shizuoka University, Shizuoka 422-8529, Japan, ²Department of Biology, Faculty of
10 Science, Shizuoka University, Shizuoka 422-8529, Japan, ³ Department of Agricultural
11 Science, Gifu University, Gifu 501-1193, Japan, ⁴ Institute for Genetic Research and
12 Biotechnology, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan,
13 ⁵Department of Animal Science, McGill University, Lakeshore Rd,
14 Ste-Anne-De-Bellevue, QC, Canada, ⁶ Graduate School of Bioagricultural Sciences,
15 Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

16
17 **Running head:** Sperm proteasome in birds

18
19 **Keywords:** Sperm; proteasome; ZP1; fertilization; Japanese quail

20
21 **Corresponding author:** Tomohiro Sasanami

22 Email address: atsasan@ipc.shizuoka.ac.jp, Tel & Fax: +81-54-238-4526,

23 Mailing address: Department of Applied Biological Chemistry, Faculty of Agriculture,
24 Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan.

25

1

2 **Abstract**

3 At the time of fertilization, the extracellular matrix surrounding avian oocytes,
4 termed the perivitelline membrane (pvm) is hydrolyzed by a sperm-borne protease,
5 although the actual protease that is responsible for the digestion of the pvm remains to
6 be identified. Here, we show evidence that the ubiquitin-proteasome system is
7 functional in the fertilization of Japanese quail. The activities for the induction of the
8 acrosome reaction and binding to ZP3 as revealed by ligand blotting of purified serum
9 ZP1 are similar to those of pvm ZP1. Western blot analysis of purified ZP1 and ZP3
10 by the use of the anti-ubiquitin antibody showed that only pvm ZP1 was reactive to the
11 antibody. In vitro penetration assay of the sperm on the pvm indicated that fragments
12 of ZP1 and intact ZP3 were released from the pvm. Western blot analysis using the
13 anti-20S proteasome antibody and ultrastructural analysis showed that immunoreactive
14 proteasome was localized in the acrosomal region of the sperm. Inclusion of specific
15 proteasome inhibitor MG132 in the incubation mixture, or depletion of extracellular
16 ATP by the addition of apyrase, efficiently suppressed the sperm perforation of the pvm.
17 These results demonstrate for the first time that the sperm proteasome is important for
18 fertilization in birds, and that the extracellular ubiquitination of ZP1 might occur during
19 its transport via blood circulation.

20

1 **Introduction**

2 In fertilization, the male and female gamete, sperm and oocyte, unite to produce a
3 zygote, a fertilized egg. Fertilization is achieved with multiple steps under precise and
4 ingenious regulation. These steps include sperm chemotaxis/activation, sperm-egg
5 binding, induction of the acrosome reaction (AR) of sperm, and membrane fusion of the
6 gametes (Florman & Ducibella, 2006). When a sperm encounters the oocyte, it must
7 pass through the extracellular coat overlaid on the surface of the oolemma, which is
8 called the zona pellucida (ZP) in mammals and the vitelline envelope in marine
9 invertebrates or in amphibians. It is believed that the sperm-borne protease hydrolyzes
10 the egg extracellular coat and that the hole produced by the protease is the path for
11 sperm penetration (Florman & Ducibella, 2006). It had long been believed that, in
12 mammalian species, the sperm acrosin, a non-ATP-dependent serine-protease localized
13 in the sperm head, is indispensable for penetration of the sperm (McRorie & Williams,
14 1974). However, Baba *et al.* demonstrated that acrosin-null male mice produce sperm
15 normal in motility and are fertile; therefore, acrosin is not essential for fertilization, at
16 least in mouse (Baba *et al.*, 1994). At present, acrosin can be interpreted as being
17 responsible for the dispersal of the acrosomal contents in the acrosome reaction
18 (Yamagata *et al.*, 1998). Sawada and his colleague reported that in marine
19 invertebrate such as ascidians and sea urchins, sperm proteasomes is responsible for
20 sperm penetration of the vitelline envelope, and the proteasome is a lytic agent called
21 lysin, which is essential for disintegration of the egg extracellular matrix (Sawada *et al.*,
22 2002a; Sakai *et al.*, 2003; Yokota & Sawada, 2007a). A similar conclusion was drawn
23 from mammalian fertilization, including that in mice, pigs and man (Pasten *et al.*, 2005;
24 Zimmerman *et al.*, 2011; Kong *et al.*, 2009).

25 In avian species, the perivitelline membrane (pvm), which is the egg envelope
26 homologous to ZP in mammals, is observed in follicles between granulosa cells and the

1 ovum before ovulation (Wyburn *et al.*, 1965). Fertilization occurs within the
2 infundibular portion of the oviduct, and only the pvm encloses the oocyte at the time of
3 fertilization. Sperm–egg interaction in avian species can be measured *in vitro* as the
4 ability of the sperm to hydrolyze a hole in the pvm (Robertson *et al.*, 2000). This hole
5 is relatively large in size (approximately 9-20 μm in diameter) in comparison with the
6 outer diameter of the sperm head (0.6 μm), and can clearly be observed in a light
7 microscope at low magnification. By using this *in vitro* assay, we demonstrated that
8 an *N*-glycan present on ZP1, one of the major components of pvm that is produced in
9 the liver under estrogen control (Sasanami *et al.*, 2003), has the ability to induce AR in
10 Japanese quail (Sasanami *et al.*, 2007). More recently, we found that the 45-kDa
11 sperm acrosin localized on the sperm plasma membrane is responsible for binding the
12 sperm to the pvm of the oocyte, but is not important for digestion of the pvm
13 glycoproteins (Sasanami *et al.*, 2011). These results indicate that the actual lysin that
14 functions during avian fertilization is not acrosin but another protease existing in the
15 sperm. Additionally, we found that ZP1, which circulates in the bloodstream received
16 some modifications during transport to the pvm. It is from the fact that when ZP1
17 purified from the serum of laying quail was injected into different birds, the signal of
18 exogenous ZP1 was detected in the pvm, whereas ZP1 derived from the pvm failed to
19 incorporate into the pvm (Kinoshita *et al.*, 2008). In this report, we demonstrate for
20 the first time that the ubiquitin-proteasome system functions in avian fertilization.
21 Moreover, we also show that the target protein of sperm proteasome is ZP1 in quail, and
22 suggest that the ubiquitination of ZP1 might take place during the transport from the
23 bloodstream to the pvm matrix.

24

25 **Results**

26 ***Differences in biological activities and biochemical properties between serum and***

1 *pvm ZP1s*

2 In order to differentiate the biological activities and biochemical properties of
3 purified serum and pvm ZP1, we compared the potential of the protein to induce an
4 acrosome reaction of ejaculated sperm. As shown in Figure 1A, serum and pvm ZP1
5 possess similar activities for the induction of acrosomal exocytosis. In addition, when
6 the purified proteins were incubated with [³H] labelled ZP3 to compare its binding to
7 the ZP3, their abilities to interact with ZP3 did not differ markedly from each other
8 (Figure 1B). The apparent molecular weight as determined by SDS-PAGE as well as
9 the reactivities of the proteins to our anti-ZP1 antiserum also did not differ much, but
10 we found that only the pvm ZP1 reacted well to the anti-Ub antibody (Figure 2). This
11 immunoreaction is specific because the serum ZP1 did not react with the anti-Ub
12 antibody and nonspecific mouse IgG failed to react with the purified ZP1s. On the
13 other hand, the purified ZP3 treated with similar conditions did not react with the
14 anti-Ub antibody.

15 *Degradation of ZP1 by ejaculated sperm*

16 The fact that pvm ZP1 is ubiquitinated led us to hypothesize that the ubiquitinated
17 pvm ZP1 might be degraded by sperm-derived proteasome during fertilization. To
18 confirm this hypothesis, we incubated the isolated pvm with ejaculated sperm, and the
19 solubilized materials released from the pvm during incubation were analyzed by
20 Western blotting. As shown in Figure 3A, the 30-min incubation with the sperm
21 successively released the fragments, which reacted with anti-ZP1 antiserum. These
22 fragments are thought to be derived from pvm during incubation with the sperm because
23 no band was seen in the absence of either sperm or pvm in the reaction mixture. When
24 the same sample was detected with the anti-ZP3 antibody, immunoreactive 35-kDa ZP3
25 was detected when the pvm and sperm were co-incubated. However, it should be
26 noted that only intact 35-kDa ZP3 was detected in the sample. These results indicate

1 that the fragment of the ZP1 as well as intact ZP3 is released from the pvm during
2 co-incubation with the sperm, and that this phenomenon might be related to the hole
3 formation during quail fertilization. In the next experiments, we compared the
4 susceptibility of the serum and pvm ZP1 to break down by the sperm. As shown in
5 Figure 3B, the intensity of the 97-kDa band of purified pvm ZP1 progressively
6 decreased during incubation, and completely disappeared after 60 min of incubation.
7 However, the band of serum ZP1 remained after 60 min exposure to the sperm.

8 ***Detection of proteasome in quail sperm***

9 In the next set of experiments, we tested whether a proteasome exists in the quail
10 spermatozoa. To achieve this goal, we detected the sperm extracts by Western blotting
11 by using anti-20S proteasome antibodies (Figure 4). The membrane fraction prepared
12 from the cavitated sperm by ultracentrifugation was shown to contain immunoreactive
13 materials that reacted with anti- α 5 (arrow in panel A, group -, lane 1), anti- α 6 (arrow in
14 panel B, group -, lane 1) and anti- α 7 antibodies (arrow in panel C, group -, lane 1).
15 The cytosol fraction also included the immunoreactive protein that appears at the same
16 location in SDS-PAGE (lane 2 of group - in each panel). When each antibody was
17 pre-absorbed by its respective antigen, the bands migrating to around 30 kDa in both the
18 membrane and the cytosol disappeared (lanes 1 and 2 of group + in each panel). These
19 results suggest that the 30-kDa bands reacting with anti- α 5, anti- α 6 or anti- α 7
20 antibodies are authentic subunits of the proteasome in quail spermatozoa. Because the
21 bands migrating around 17 kDa that reacted with anti- α 6 were not reduced by antigen
22 absorption (panel B, group +), this protein appears to react non-specifically with the
23 antibody. To analyze more precisely the localization of the proteasome, we performed
24 immunoelectron microscopy for the detection of the antigen in ultra-thin sections of the
25 sperm (Figure 5). The immunogold particles were distributed in the acrosomal region
26 of the sperm head as well as in the nucleus when the specimens were incubated with

1 anti- α 5 antibody (panel A). No such accumulation of gold particles in the acrosome
2 region was observed when the specimens were incubated with anti- α 5 antibody that had
3 been preabsorbed with the antigen protein, but the signals persisted in the nucleus of the
4 sperm (panel B). The immunogold accumulation in the nucleus was found to be a
5 non-specific signal due to the reactivities of the secondary antibody used because this
6 signals also found when the first antibody had been omitted (data not shown). These
7 observations demonstrated that the antigen reactive to the anti- α 5 antibody localizes in
8 the acrosome region of the sperm head. Similar results were obtained when the
9 specimens were reacted with anti- α 7 antibody (data not shown).

10

11 *Effects of proteasome inhibitor and apyrase on the hole formation on the pvm by the* 12 *sperm*

13 To provide further support to the idea that sperm proteasome is crucial for degrading
14 the pvm by sperm, we examined whether MG132, a proteasome inhibitor, and apyrase,
15 which catalyzes the hydrolysis of ATP and ADP, inhibits the *in vitro* perforation of the
16 pvm by the sperm. When the pvm was incubated with the sperm in the presence of
17 MG132, only few perforations were observed, whereas many holes were observed in
18 the case of the vehicle control as well as the pvm that had been incubated in the
19 presence of E64d, a cysteine protease inhibitor (Figure 6). In addition to these
20 inhibitory effects on hole formation, apyrase also potently inhibited sperm perforation
21 *in vitro* (Figure 7). These results strongly suggest that sperm proteasome plays a
22 crucial role in the degradation of the pvm during fertilization.

23

24 **Discussion**

25 In the present study, we demonstrated for the first time that the degradation of the
26 pvm by sperm to produce holes in it during avian fertilization is mediated, at least, in

1 part, by the ubiquitin-proteasome system. This is strongly supported by the following
2 findings. First, the ejaculated spermatozoa contain proteasome and that this
3 proteasome is localized in the acrosomal region of the sperm head. Second, ZP1, a
4 major constituent of the pvm, is reactive to anti-ubiquitin antibody, and the ejaculated
5 sperm possess the potencial to break down ZP1. However, the sperm fail to
6 decompose ZP3, another major component of pvm, which appear not to undergo
7 ubiquitin modification. Third, the degradation of serum ZP1 that does not carry the
8 ubiquitin is retarded compared with that of pvm ZP1. Fourth, sperm protease activity
9 to degrade the pvm is strongly disturbed by MG132, a potent proteasome inhibitor, but
10 is not affected by E64d, a cysteine protease inhibitor. Fifth, when the extracellular
11 ATP is depleted by the addition of apyrase to the incubation mixture, hole formation is
12 rarely observed. As described here, many lines of evidence obtained in this study
13 suggest that the ubiquitin-proteasome system is pivotal to making holes in the pvm and
14 subsequent sperm penetration in quail fertilization. It was reported in the chicken that
15 acrosin, an acrosomal serine protease, plays an important role in fertilization, and is
16 believed to be involved in hole formation on the pvm during fertilization (Richardson *et*
17 *al.*, 1992; Slowinska *et al.*, 2010). In our previous study, we found that sperm lysates
18 have the potencial to degrade the gelatin using zymography (Sasanami *et al.*, 2011).
19 However, anti-acrosin monoclonal antibody failed to inhibit protease activity, whereas
20 this antibody effectively disturbed the binding of the sperm to the pvm, indicating that
21 acrosin plays a role in sperm-egg recognition in quail fertilization (Sasanami *et al.*,
22 2011). In addition, the inhibition of the sperm penetration by apyrase also suggested
23 that an ATP-dependent protease like proteasome, but not acrosin, participates in the
24 process of the hydrolysis of the pvm.

25 In this study, we found that sperm degraded ZP1, but not ZP3, in the *in vitro*
26 penetration assay, and that this result demonstrates that the target for the sperm

1 proteasome is ZP1 in avian species. It should be noted that the immunoreactive signal
2 of the intact 35-kDa ZP3 was detected in the sample, indicating that ZP3 was also
3 released from the pvm, but failed to be degraded. Although the target site of the
4 sperm proteasome for ZP1 is currently unknown, we previously reported that the
5 binding of ZP1 and ZP3 for egg envelope formation is mediated by the zona pellucida
6 domain, a hallmark structure of ZP protein. We think that ZP3 might be released from
7 the pvm as the ZP1-ZP3 complex.

8 The timing of the ubiquitination of the ZP proteins differs in different animals.
9 For example, Hrc70, a sperm receptor in *H. roretzi*, was found to be ubiquitinated
10 extracellularly by sperm-derived ubiquitination enzymes at the time of fertilization, and
11 the ubiquitinated Hrc70 protein is degraded with sperm-borne proteasome (Sawada *et*
12 *al.*, 2002a). In contrast, mammalian zona pellucida protein, including that in mice,
13 was ubiquitinated during the process of biosynthesis, and the intracellular ubiquitination
14 of the ZP protein has been proposed (Sutovsky *et al.*, 2004). Similar intracellular
15 ubiquitination of the egg coat protein was observed in sea urchins (Yokota & Sawada,
16 2007a). The site of ubiquitination of ZP1 in quail is unclear. However, ZP1 might
17 be modified extracellularly during transport in the bloodstream to the pvm. In electron
18 microscopic analysis, a coarse cellular junction between the granulosa cells has been
19 observed (Bellairs, 1965), and very low-density lipoprotein and vitellogenin, a precursor
20 protein of egg yolk, pass through these gaps (Perry & Gilbert, 1979). The ZP1 protein
21 probably also passes through the gap between the granulosa cells, and the ubiquitination
22 might take place during the transport. Although the possibility cannot be completely
23 ruled out, the intracellular ubiquitination of ZP1 could not have occurred because the
24 lysate obtained from granulosa and theca cells does not contain immunoreactive
25 materials against the anti-ZP1 antibody (data not shown). Identifying the site of
26 ubiquitination and the ubiquitination enzyme responsible for ZP1 modification remains

1 to be achieved in a future study.

2 In our immunoelectron microscopy tests, immunoreactive proteasome localized
3 in the acrosome of the sperm head. During the acrosome reaction in quail, the
4 acrosomal cap, which is 4 μ m in size, comes off from the head, and the acrosomal
5 contents are thought to be released. Given this, the proteasome that is released from
6 the sperm during the acrosome reaction might degrade the ubiquitinated-ZP1 in
7 fertilization. In the mouse, Inoue *et al.* (2011) clearly demonstrated that the
8 acrosome-reacted sperm recovered from the perivitelline space can penetrate the zona
9 pellucida again, and can fertilize to produce healthy pups. In addition, Jin *et al.* (2011)
10 recently showed evidence that most fertilizable sperm is not intact sperm, but acrosome
11 reacted sperm by live-imaging of the sperm-egg interaction. From these observations,
12 the acrosomal contents including protease are not that important for achieving
13 fertilization in mice. In birds, the holes that form in the pvm due to sperm during
14 fertilization are huge compared to those in the mammalian zona pellucida, the fibers in
15 the holes disappear, and the edges of the holes appear to be smooth (Takeuchi *et al.*,
16 2001). The holes do not develop only by the mechanical force generated by sperm
17 motility, but also by other means, namely, a protease. We do not know whether
18 acrosome-reacted sperm can hydrolyze pvm owing to the technical difficulty of
19 collecting acrosome-reacted sperm without any contamination by intact sperm; however,
20 on the basis of the observations mentioned above, we believe that an acrosome-derived
21 protease like proteasome is important for the hydrolysis of pvm in birds based on the
22 observations mentioned above.

23 In Western blot analysis of the cavitated sperm, the immunoreactive materials
24 recognized by the anti-20S proteasome subunit were localized in both the cytosol and
25 membrane fractions. This result indicates the presence of membrane-associated
26 proteasome in the sperm. Sawada and his colleagues also reported that sperm

1 proteasome appears to be localized on the sperm surface because cell-impermeable
2 NHS-LC-biotin is capable of labeling the sperm proteasome (Sawada *et al.*, 2002b).
3 More recently, Yokota *et al.* (2011) demonstrated that the 16 C-terminal residues of the
4 sperm proteasome $\alpha 6$ subunit are post-translationally and proteolytically processed in a
5 testis-specific manner in *H. roretzi*, and the possible involvement of this processing in
6 the extracellular transport of the sperm proteasome has been suggested. Taken
7 together with our experimental data, we think that the proteasome in quail sperm might
8 localize not only inside the acrosomal matrix, but also on the surface of the cells. The
9 specific role of proteasome that might be exposed to the outside on the surface of the
10 sperm remains to be studied.

11 In conclusion, we demonstrated for the first time that the ubiquitin-proteasome
12 system functions in avian fertilization. The target protein for sperm proteasome was
13 found to be ubiquitinated pvm ZP1, and the presence of the post-translational
14 ubiquitination of ZP1 during transport was suggested. Although the timing of the
15 ubiquitination of the target protein differs in different species, the ubiquitin-proteasome
16 system might be universally conserved in animal fertilization. The avian system is a
17 beneficial model for studying the role of sperm proteasome in fertilization because
18 avian sperm can produce a huge hole on the pvm, and large quantities of the target
19 protein, ZP1, can be isolated from a large oocyte.

20

21 **Materials and Methods**

22 *Animals and tissue preparation*

23 Male and female Japanese quail, *Coturnix japonica*, 15-30 weeks of age (Kato-farm,
24 Toyohashi, Japan), were maintained individually under a photoperiod of 14L: 10D (with
25 the light on at 0500) and were provided with water and a commercial diet
26 (Tokai-Hokuriku Nosan, Chita, Japan) *ad libitum*. The female animals were

1 decapitated and the largest preovulatory follicles were dissected. The granulosa layer
2 from the largest preovulatory follicles was isolated as a sheet of granulosa cells
3 sandwiched between the pvm and basal laminae, as previously described (Gilbert *et al.*,
4 1977). The pvm was isolated according to a procedure described by Sasanami *et al.*
5 (Sasanami *et al.*, 2002). The isolated pvm was used for in vitro assay for sperm-egg
6 interaction. A proctodeal gland secretion was obtained manually from male quail as
7 meringue-like foam. This foam was then centrifuged at 10,000 g for 10 min, and the
8 supernatants collected were stored as a proctodeal gland secretion at -80 °C until use.

9 All experimental procedures for the care and use of animals in the present study
10 were approved by the Animal Care Committee of Shizuoka University (approval
11 number, 22-12).

12 ***Purification of serum and pvm ZP1***

13 The pvm lysate was separated by one-dimensional SDS-PAGE, performed as
14 described by Laemmli (Laemmli, 1970), under non-reducing conditions on 12% (w/v)
15 polyacrylamide separating gel. The samples (750 µg of protein per gel) were applied
16 to 5% (w/v) stacking gel without a comb for lane casting. After electrophoresis, the
17 gel was stained with Copper Stain (Bio-Rad Laboratories, Hercules, CA, USA), and the
18 97-kDa ZP1 band was excised. The proteins were eluted by incubating the gel slices
19 with 0.1% (w/v) SDS buffered at pH 8.0 with 100 m mol l⁻¹ Tris-HCl overnight at 4 °C
20 with constant shaking. The eluent was then extensively dialyzed against water,
21 lyophilized, and dissolved in PBS. The protein concentration of the sample was
22 measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

23 To prepare the affinity gel for the separation of serum ZP1, the IgG fractionated
24 from anti-ZP1 antiserum (Kinoshita *et al.*, 2008) with a HiTrap Protein A FF affinity
25 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) that had been covalently
26 coupled to NHS-activated sepharose (Amersham Pharmacia Biotech) according to the

1 manufacturer's instructions. The serum of laying birds was incubated with the
2 affinity gel for 16 h at 4 °C. After extensive washing with PBS, the gel was eluted
3 with elution buffer (1 mol l⁻¹ CH₃COOH, 0.1 m mol l⁻¹ glycine, pH 2.5) and the eluent
4 containing serum ZP1 was recovered. The 97-kDa serum ZP1 was purified with the
5 same procedure as that used to purify pvm ZP1. The purity of the ZP1s was confirmed
6 by CBB staining after separation of the protein by SDS-PAGE.

7 ***Semen collection and preparation***

8 Ejaculated semen was obtained from male quail prior to mating according to the
9 procedure of Kuroki & Mori (Kuroki and Mori, 1997). Semen obtained from two to
10 three males was suspended in Hanks' balanced salt solution (HBSS) containing 1.25 m
11 mol l⁻¹ of CaCl₂ and 0.1% (v/v) of proctodeal gland secretion. We added proctodeal
12 gland secretion to the incubation mixture since it inhibits agglutination of the quail
13 sperm. The concentrations of sperm were measured with a hemocytometer and the
14 sperm viabilities were determined using the LIVE/DEAD sperm viability kit according
15 to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). In all the
16 experiments, sperm were incubated at 39 °C.

17 To obtain the sperm membrane or cytosol fraction, the ejaculates were washed 3
18 times with ice-cold PBS with repeated centrifugation at 800 x g for 5 min at 4°C, and
19 the final pellet was suspended in buffer containing 150 m mol l⁻¹ NaCl, 20 m mol l⁻¹
20 HEPES (pH 7.4). The suspension was cavitated with a cell disruptor (Parr Instrument
21 Company, Moline, IL, USA) at 400 psig, and the cellular debris was removed by
22 centrifugation at 10,000 x g for 10 min. The supernatants were further centrifuged at
23 100,000 x g for 30 min, and the supernatants (cytosol) and the precipitates (membrane)
24 stored at -80°C until use.

25 ***In vitro assay for sperm-egg interaction***

26 A piece of pvm, approximately 8 mm in diameter, was incubated in a micro-test tube

1 with 0.5ml of sperm suspension at 1×10^7 sperm /ml in HBSS at 39 °C for 0 to 60 min.
2 After incubation, the reaction was terminated by placing the tube on ice and the pvm
3 was washed 3 times with ice-cold PBS, then transferred onto a glass slide and stained
4 with Schiff's reagent after fixation with 3.7 % (v/v) formaldehyde in PBS. The holes
5 that formed on the pvm in the x200 field were photographed in a light microscope (BX
6 51, Olympus Optics, Tokyo, Japan), and the number of holes per 0.15mm^2 was
7 calculated using image J software (version 1.44). At least five areas were randomly
8 selected for enumeration of perforations. For the analysis of the interaction of purified
9 ZP1 with sperm, ejaculated sperm at 1×10^7 sperm /ml in HBSS was mixed with equal
10 amounts of the purified ZP1 solution diluted in HBSS (50 $\mu\text{g/ml}$), and incubated as
11 described above.

12 To observe the inhibitory activity of the anti-20S proteasome antibodies, the pvm
13 was added into a tube containing either anti-20S proteasome antibody (anti- $\alpha 5$ or
14 anti- $\alpha 7$) (Wakata *et al.*, 2004) or normal guinea pig serum diluted 1:200 with HBSS,
15 and sperm suspension was then added at 1×10^7 sperm /ml. The effects of 50 μM
16 carbobenzoxy-Leu-Leu-Leu-H (MG132, sigma), a proteasome inhibitor, 50 μM
17 [2S-3S-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d, sigma), a
18 cysteine protease inhibitor, or 200 units/ml apyrase (Sigma) that catalyses the
19 hydrolysis of ATP and ADP on the sperm perforation of the pvm were also tested as
20 described above. The concentration of MG132 and E64d were adopted from Yokota
21 and Sawada (Yokota & Sawada, 2007b). Appropriate vehicle (dimethylsulfoxide for
22 MG132 and E54d, saline for apyrase) was included in the incubation mixture for the
23 control experiments and the final concentration of each solvent was adjusted to 0.1%.

24 For evaluation of the acrosomal status of the sperm, the ejaculated spermatozoa was
25 incubated with or without purified ZP1 for 10 min as described previously (Sasanami *et*
26 *al.*, 2007).

1 ***Gel electrophoresis, Western blot and ligand blot analysis***

2 For Western blotting, the cavitated sperm proteins separated by SDS-PAGE were
3 electrotransferred to a PVDF membrane (Immobilon-P, Millipore Bedford, MA, USA)
4 (Matsudaira, 1987). The membrane incubated with anti-20S proteasome antibody
5 (anti- α 5 or anti- α 7) or normal guinea pig serum diluted 1:2,000 followed by
6 horseradish peroxidase-conjugated anti-guinea pig IgG (Cappel, Durham, NC, USA) as
7 a secondary antibody was visualized by means of a chemiluminescent technique
8 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For detection of ubiquitinated
9 ZP proteins, the membrane that immobilized the purified ZP1 or ZP3 was reacted with
10 anti-ubiquitin antibody (P4D1, Santa Cruz Biotechnology, Santa Cruz, CA, USA),
11 which reacts with free ubiquitin, poly-ubiquitinated and mono-ubiquitinated proteins.
12 The signals were detected with horseradish peroxidase-conjugated anti-mouse IgG
13 (Cappel) as a secondary antibody. The immuno blot for ZP1 and ZP3 was performed
14 as described previously (Sasanami *et al.*, 2002; Sasanami *et al.*, 2006). For ligand blot
15 analysis a PVDF strip electrotransferred with pvm lysate or purified ZP1 was incubated
16 with [³H] labeled ZP3 as described previously (Ohtsuki *et al.*, 2004).

17 ***Immunoelectron microscopy***

18 Ejaculated sperm embedded in 3% (w/v) agarose were fixed with 2.5% (v/v)
19 glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4 °C. The specimens
20 were embedded in Lowicryl K₄M resin (Polysciences, Warrington, PA, USA). Thin
21 sections were first treated with anti- α 5 antibody (1:20) or anti- α 5 antibody preabsorbed
22 with antigen protein (1:20), and then with a gold-conjugated goat antiserum against
23 guinea pig IgG (1:10) (E-Y Laboratories, San Mateo, CA, USA). The sections were
24 stained with uranyl acetate and observed with a model H-8000 electron microscope
25 (Hitachi, Tokyo, Japan)

26

1 **Declaration of interest**

2 The authors declare that there is no conflict of interest that could be perceived as
3 prejudicing the impartiality of the research reported.

4 **Funding**

5 This work was supported by financial support from a Grant-in-Aid for Scientific
6 Research (B) (General) (24380153 to TS), a Grant-in-Aid for Scientific Research on
7 Innovative Areas (24112710 to TS), and the Sumitomo Foundation (101069 to TS).

8

9 **Acknowledgements**

10 The authors are grateful to Mr. Y. Ogasa, Dr. K. Yoshimatsu and Miss M. Matsuzaki
11 for technical assistance.

1 **References**

- 2 **Baba T, Azuma S, Kashiwabara S & Toyoda Y** 1994 Sperm from mice carrying a
3 targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect
4 fertilization. *Journal of Biological Chemistry* **269** 31845-31849.
- 5 **Bellairs R** 1965 The relationship between oocyte and follicle in the hen's ovary as
6 shown by electron microscopy. *Journal of Embryology & Experimental Morphology* **13**
7 215-233.
- 8 **Florman HM & Ducibella T** 2006 In *Physiology of Reproduction*, 3 edn, Vol 1, pp
9 55–112. Ed. JD Neill. St Louis, MO, USA: Elsevier Academic Press.
- 10 **Gilbert AB, Evans AJ, Perry MM & Davidson MH** 1977 A method for separating
11 the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of
12 the domestic fowl (*Gallus domesticus*). *Journal of Reproduction & Fertility* **50**
13 179–181.
- 14 **Inoue N, Satouh Y, Ikawa M, Okabe M & Yanagimachi R** 2011 Acrosome-reacted
15 mouse spermatozoa recovered from the perivitelline space can fertilize other eggs.
16 *Proceedings of the National Academy of Sciences of the United States of America* **108**
17 20008-20011.
- 18 **Jin M, Fujikawa E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K &**
19 **Hirohashi N** 2011 Most fertilizing mouse spermatozoa begin their acrosome reaction
20 before contact with the zona pellucida during in vitro fertilization. *Proceedings of the*
21 *National Academy of Sciences of the United States of America* **108** 4892-4896.
- 22 **Kinoshita M, Mizui K, Ishiguro T, Ohtsuki M, Kansaku N, Ogawa H, Tsukada A,**
23 **Sato T & Sasanami T** 2008 Incorporation of ZP1 into perivitelline membrane after in
24 vivo treatment with exogenous ZP1 in Japanese quail (*Coturnix japonica*). *FEBS*
25 *Journal* **275** 3580-3589.
- 26 **Kong M, Diaz ES & Morales P** 2009 Participation of the human sperm proteasomes in

1 the capacitation process and its regulation by protein kinase A and tyrosine kinase.
2 *Biology of Reproduction* **80** 1026-1035.

3 **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of
4 bacteriophage T4. *Nature* **227** 680-685.

5 **Matsudaira P** 1987 Sequence from picomole quantities of proteins electroblotted onto
6 polyvinylidene difluoride membranes. *Journal of Biological Chemistry* **262**
7 10035-10038.

8 **McRorie RA & Williams WL** 1974 Biochemistry of mammalian fertilization. *Annual*
9 *Review of Biochemistry* **43** 777-803.

10 **Ohtsuki M, Hanafy AM, Mori M & Sasanami T** 2004 Involvement of interaction of
11 ZP1 and ZPC in the formation of quail perivitelline membrane. *Cell & Tissue Research*
12 **318** 565-570.

13 **Pasten C, Morales P & Kong M** 2005 Role of the sperm proteasomes during
14 fertilization and gamete interaction in the mouse. *Molecular Reproduction and*
15 *Development* **71** 209-219.

16 **Perry MM & Gilbert AB** 1979 Yolk transport in the ovarian follicle of the hen (*Gallus*
17 *domesticus*): lipoprotein-like particles at the periphery on the oocyte in the rapid growth
18 phase. *Journal of Cell Science* **39** 257-272.

19 **Richardson ME, Korn N, Bodine AB & Thurston RJ** 1992 Kinetic and inhibition
20 studies with turkey acrosin. *Poultry Science* **71** 1789-1793.

21 **Robertson L, Wishart GJ & Horrocks AJ** 2000 Identification of perivitelline N-linked
22 glycans as mediators of sperm-egg interaction in chickens. *Journal of Reproduction &*
23 *Fertility* **120** 397-403.

24 **Sakai N, Sawada H & Yokosawa H** 2003 Extracellular ubiquitin system implicated in
25 fertilization of the ascidian, *Halocynthia roretzi*: isolation and characterization.
26 *Developmental Biology* **264** 299-307.

1 **Sasanami T, Pan J, Doi Y, Hisada M, Kohsaka T & Toriyama M** 2002 Secretion of
2 egg envelope protein ZPC after C-terminal proteolytic processing in quail granulosa
3 cells. *European Journal of Biochemistry* **269** 2223-2231.

4 **Sasanami T, Pan J & Mori M** 2003 Expression of perivitelline membrane
5 glycoprotein ZP1 in the liver of Japanese quail (*Coturnix japonica*) after in vivo
6 treatment with diethylstilbestrol. *Journal of Steroid Biochemistry & Molecular Biology*
7 **84** 109-116.

8 **Sasanami T, Ohtsuki M, Ishiguro T, Matsushima K, Hiyama G, Kansaku N, Doi Y**
9 **& Mori M** 2006 Zona pellucida domain of ZPB1 controls specific binding of ZPB1 and
10 ZPC in Japanese quail (*Coturnix japonica*). *Cells, Tissues, Organs* **183** 41-52.

11 **Sasanami T, Murata T, Ohtsuki M, Matsushima K, Hiyama G, Kansaku N & Mori**
12 **M** 2007 Induction of sperm acrosome reaction by perivitelline glycoprotein ZP1 in
13 Japanese quail (*Coturnix japonica*). *Reproduction* **133** 41-49.

14 **Sasanami T, Yoshizaki N, Dohra H & Kubo H** 2011 Sperm acrosin is responsible for
15 the sperm binding to the egg envelope during fertilization in Japanese quail (*Coturnix*
16 *japonica*). *Reproduction* **142** 267-276.

17 **Sawada H, Sakai N, Abe Y, Tanaka E, Takahashi Y, Fujino J, Kodama E,**
18 **Takizawa S & Yokosawa H** 2002a Extracellular ubiquitination and
19 proteasomes-mediated degradation of the ascidian sperm receptor. *Proceedings of the*
20 *National Academy of Sciences of the United States of America* **99** 1223-1228.

21 **Sawada H, Takahashi Y, Fujino J, Flores SY & Yokosawa H** 2002b Localization
22 and roles in fertilization of sperm proteasomes in the ascidian *Halocynthia roretzi*.
23 *Molecular Reproduction & Development* **62** 271-276.

24 **Slowinska M, Olczak M, Liszewska E, Watorek W & Ciereszko A** 2010 Isolation,
25 characterization and cDNA sequencing of acrosin from turkey spermatozoa.
26 *Comparative Biochemistry & Physiology, Part B* **157** 127-136.

1 **Sutovsky P, Manandhar G, Mccauley TC, Caamano JN, Sutovsky M, Thompson**
2 **WE & Day BN** 2004 Proteasomal interference prevents zona pellucida penetration and
3 fertilization in mammals. *Biology of Reproduction* **71** 1625-1637.

4 **Takeuchi Y, Cho R, Iwata Y, Nishimura K, Kato T, Aoki N, Kitajima K, Matsuda**
5 **T** 2001 Morphological and biochemical changes of isolated chicken egg-envelope
6 during sperm penetration: degradation of the 97-kilodalton glycoprotein is involved in
7 sperm-driven hole formation on the egg-envelope. *Biology of Reproduction* **64** 822-830.

8 **Yamagata K, Murayama M, Okabe M, Toshimori K, Nakanichi T, Kashiwabara S**
9 **& Baba T** 1998 Acrosin accelerates the dispersal of sperm acrosomal proteins during
10 acrosome reaction. *Journal of Biological Chemistry* **273** 10470-10474.

11 **Yokota N & Sawada H** 2007a Sperm proteasomes are responsible for the acrosome
12 reaction and sperm penetration of the vitelline envelope during fertilization of the sea
13 urchin *Pseudocentrotus depressus*. *Developmental Biology* **308** 222-231.

14 **Yokota N & Sawada H** 2007b Effects of proteasomes inhibitors on fertilization of the
15 sea urchin *Anthocidaris crassispina* *Biological & Pharmaceutical Bulletin* **30**
16 1332-1335.

17 **Yokota N, Kataoka Y, Hashii N, Kawasaki N & Sawada H** 2011 Sperm-specific C
18 terminal processing of the proteasomes PSMA1/ α 6 subunit. *Biochemical & Biophysical*
19 *Research Communications* **410** 809-815.

20 **Wakata Y, Tokumoto M, Horiguchi R, Ishikawa K, Nagahama Y & Tokumoto T**
21 2004 Identification of α -type subunits of the *Xenopus* 20S proteasomes and analysis of
22 their changes during the meiotic cell cycle. *BMC Biochemistry* **5**: 18.11. **Wyburn GM,**
23 **Aitken RNC & Johnston HS** 1965 The ultrastructure of the zona radiata of the ovarian
24 follicle of the domestic fowl. *Journal of Anatomy* **99** 469-484.

25 **Zimmerman, SW, Manandhar G, Yi Y, Gupta SK, Sutovsky M, Odhiambo JF,**
26 **Powell D, Miller DJ & Sutovsky P** 2011 Sperm proteasomes degrade sperm receptor

1 on the egg zona pellucida during mammalian fertilization. *PLoS ONE* **6** e17256.

2

3 **Figure legends**

4 **Figure 1 Comparison of biological activity of serum and pvm ZP1.** (A) The
5 ejaculated sperm were incubated in the presence or absence of purified ZP1 at 39°C for
6 30 min. After incubation, the percentage of the acrosome-reacted sperm was
7 calculated as described in Experimental procedures. Values are mean ± SD of three
8 independent experiments. (B) Ligand blot analysis of purified ZP1 with [³H] labelled-
9 ZP3. Purified serum (S-ZP1) or pvm (pvm-ZP1) ZP1 were separated by SDS-PAGE,
10 and then electro transferred onto a PVDF membrane. Binding of [³H] labeled-ZP3 to
11 ZP1 was detected by ligand blot analysis. As a positive control, pvm lysate (10
12 µg/lane) was subjected to SDS-PAGE and detected in the same manner.
13 Representative results of repeated experiments are shown. An arrow indicates the
14 radio-labelled signal of monomeric ZP1.

15

16 **Figure 2: Detection of ubiquitination of pvm-ZP1.** Purified serum (S; 5 µg/lane) or
17 pvm ZP1 (pvm; 5 µg/lane) was separated by SDS-PAGE and the protein was visualized
18 by CBB staining. The same sample (1 µg/lane) was detected with Western blotting
19 using anti-ZP1 antiserum, anti-ubiquitin antibody or normal mouse IgG. As shown in
20 the right panel, purified ZP3 (1 µg/lane) was separated with SDS-PAGE, and was
21 detected using anti-ZP3 antiserum or anti-ubiquitin antibody. Note that the
22 immunoreactive band against the anti-ubiquitin antibody was seen in the lane of
23 purified pvm ZP1, but not in the lane of the serum ZP1 or purified ZP3. Results
24 shown in the figure are representative of repeated experiments.

25

26 **Figure 3: Degradation of ZP1 by ejaculated spermatozoa.** (A) The pvm isolated

1 from the largest follicles was incubated with (+ pvm and + sperm) or without (+ pvm
2 and - sperm) ejaculated spermatozoa at 39°C for 30 min. For the negative control, only
3 sperm were incubated in the same conditions (- pvm and +sperm). After the
4 incubation, the degraded materials of the pvm were collected by centrifugation and
5 detected with Western blotting by using anti-ZP1 or anti-ZP3 antiserum. Shown are
6 representative results of repeated experiments. **(B)** 5 µg of purified pvm ZP1 or serum
7 ZP1 was incubated with ejaculated spermatozoa for 0, 10, 30 or 60 min at 39°C. After
8 the incubation, the reaction mixtures were centrifuged and the supernatants detected
9 using anti-ZP1 antiserum. An arrow indicates intact ZP1. Representative results of
10 repeated experiments are shown.

11

12 **Figure 4 Detection of proteasome in the ejaculated sperm.** The ejaculated sperm
13 suspended in the buffer were cavitated as described in Experimental procedures. After
14 sedimentation of the debris by centrifugation, the supernatants was ultracentrifuged to
15 separate the membrane (lane 1) and cytosol fractions (lane 2). Samples were then
16 separated by SDS-PAGE and detected using anti-20S proteasome $\alpha 5$ subunit antiserum
17 (group – in panel A), anti-20S proteasome $\alpha 6$ subunit antiserum (group – in panel B) or
18 anti-20S proteasome $\alpha 7$ subunit antiserum (group – in panel C). Each antiserum was
19 preabsorbed with its respective antigen for 1 hr at 4°C to prepare control antiserum
20 (group + in panels A-C). Arrows indicate the band of proteasome.

21

22 **Figure 5 Immunoelectron microscopy of ejaculated sperm.** Ultra-thin sections of
23 quail sperm were incubated with the anti-20S proteasome $\alpha 5$ subunit antiserum **(A)** or
24 normal guinea pig serum **(B)**. The accumulation of gold particles was frequently
25 found in the sperm acrosome (the acrosome located between the long arrows) when the
26 sections were incubated with antiserum, whereas no accumulation was seen on the

1 sections incubated with the antiserum, preincubated with antigen. Note that the
2 immunogold particles were localized both sections of the nucleus (short arrow), and that
3 this signal was thought to be non-specific. The representative results of repeated
4 experiments are shown. Bar represents 500 nm.

5

6 **Figure 6: Effects of proteasome inhibitor on in vitro sperm perforation.** The pvm
7 isolated from the largest follicles was incubated with ejaculated sperm in the presence
8 of dimethylsulfoxide (**A**), 50 μ M MG132, proteasome inhibitor (**B**) or 50 μ M E64d,
9 cysteine protease inhibitor (**C**) for 10 min. After the incubation, the pvm was spread
10 onto a glass slide, and observed in a light microscope. (**D**) The number of holes per
11 0.15 mm^2 was calculated from image J software, and is expressed as mean \pm SD. The
12 images shown in (A)-(C) are representative of repeated experiments. The values
13 depicted in (D) and (E) were obtained from three independent experiments. Bar
14 represents 50 μ m.

15

16 **Figure 7: Apyrase inhibits in vitro sperm perforation.** The pvm isolated from the
17 largest follicles was incubated with ejaculated sperm in the presence (**B**) or absence (**A**)
18 of apyrase (200 units/ml) for 10 min. After the incubation, the pvm was spread onto a
19 glass slide and observed in a light microscope. (**D**) The number of holes per 0.15
20 mm^2 was calculated from image J software and is expressed as mean \pm SD. The
21 images shown in (A)-(C) are representative of repeated experiments. The values
22 depicted in (D) and (E) were obtained from three independent experiments. Bar
23 represents 50 μ m.