# Sperm acrosin is responsible for the sperm binding to the egg envelope during fertilization in Japanese quail (Coturnix japonica)

**Title:** Sperm acrosin is responsible for the sperm binding to the egg envelope during fertilization in Japanese quail (*Coturnix japocica*).

**Author's names:** Tomohiro Sasanami[1], Norio Yoshizaki[2], Hideo Dohra[3] and Hideo Kubo[4]

**Addresses:** [1]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan, [2]Department of Agricultural Science, Gifu University, Gifu 501-1193, Japan, [3]Institute for Genetic Research and Biotechnology, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan, [4]Department of Medical Biology, Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo 156-8506, Japan

**Running head:** Sperm acrosin in quail

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**Corresponding author:** Tomohiro Sasanami
Email address: atsasan@ipc.shizuoka.ac.jp, Tel & Fax: +81-54-238-4526,
Mailing address: Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan.

**Abstract**

An antibody library against quail sperm plasma membrane components was established and a monoclonal antibody (mAb), which strongly inhibits sperm perforations of the perivitelline membrane (pvm) was obtained from the library. The antigen molecule of the mAb showed an apparent molecular weight of 45 kDa, and was distributed both on the surface and in the acrosomal matrix of the sperm head. Periodate oxidation revealed that the epitope of the antigen includes a sugar moiety. Tandem mass spectrometry analysis of the antigen revealed that the mAb recognizes sperm acrosin. When sodium dodecyl sulfate-solubilized pvm immobilized on a polyvinylidene difluoride membrane was incubated with sperm plasma membrane lysates, the sperm acrosin was detected on the pvm immobilized on the membrane, indicating that the sperm acrosin interacts with the components of pvm. Indeed, the mAb effectively inhibited the binding of acrosome-intact sperm to the pvm. These results indicate that the 45-kDa sperm acrosin is involved in the binding of sperm to the pvm in fertilization of Japanese quail.

## Introduction

Fertilization is the joining of two gametes, an oocyte and a sperm, and is the consequence of precisely ordered multiple steps, including sperm–egg binding, the induction of the acrosome reaction (AR) on the sperm, and the membrane fusion of the gametes. The zona pellucida (ZP), which is an extracellular matrix surrounding mammalian oocytes, plays an important role in fertilization, especially in the primary binding of the sperm and the induction of the AR as well as preventing polyspermy (Florman & Ducibella, 2006). This matrix is composed of three or four glycoproteins (*i.e.,* ZP1, ZP2, ZP3, and ZP4) in mammalian species (Litscher and Wassarman, 2007; Lefievre *et al.,* 2004; Ganguly *et al*., 2008; Hoodbhoy *et al*., 2005; Izquierdo-Rico *et al.,* 2009). In the case of non-mammalian vertebrates such as amphibians (i.e., *Xenopus laevis* and *X. tropicalis*), five ZP glycoproteins (ZPA (ZP2), ZPB (ZP1), ZPC (ZP3), ZPD (ZP4) and ZPAX) have been identified (Lindsay *et al*., 2003; Goudet *et al*., 2008). The ZP gene family proteins share a highly conserved amino acid sequence near the C-terminus called the ZP domain, consisting of about 260 amino acid residues with 8 or 10 conserved Cys residues (Bork & Sander, 1992). On the other hand, there are many reports suggesting that the sperm membrane proteins are important for sperm-zona interaction. For instance, the sperm-specific hyaluronidase PH-20 (Primakoff *et al.,* 1988) and Hyal5 (Kim *et al.,* 2005), beta-1, 4-galactosyltransferase (Miller *et al.*, 1992) as well as a secreted protein containing N-terminal Notch-like type II EGF repeats and C-terminal discoidin/ F5/8 C domains (SED1) (Ensslin and Shur, 2003) have been suggested to be involved in sperm-zona binding in mammalian species, including mice. Other factors in fertilization are thought to compensate for these factors, because disruption of each gene with homozygous null mutation did not make the animals sterile (Ikawa *et al*., 2008).

In avian species, the perivitelline membrane (pvm), which is the egg envelope

homologous to ZP in mammals, is observed in follicles between granulosa cells and ovum before ovulation (Wyburn *et al*. 1965). Fertilization occurs within the infundibulum portion of the oviduct, and only the pvm encloses the oocyte at the time of fertilization. Sperm–egg interaction in avian species can be measured *in vitro* as the ability of the sperm to hydrolyze a small hole in the pvm (Robertson *et al.,* 1997; Kuroki and Mori, 1997). Results of this *in vitro* assay suggested that the *N*-glycans of the pvm play an indispensable role in sperm-egg interaction and the induction of the AR in domestic fowl (Horrocks *et al.,* 2000; Robertson *et al.,* 2000). Recently, we demonstrated that an *N*-glycan present on ZP1, one of the major pvm components that is produced in the liver under estrogen control (Sasanami *et al.,* 2003), has the ability to induce the AR in Japanese quail (Sasanami *et al*., 2007). However, identification of the complementary molecules responsible for the sperm-egg interaction in birds, including the components that interact with ZP1 on the surface of the sperm, remains to be accomplished.

Recently, by the aid of an antibody library raised against the sperm plasma membrane of *X. laevis* (Nagai *et al*., 2009), Kubo *et al.* (2010) identified a component that can bind to the vitelline envelope (VE) using a newly developed method, the dot blot assay. As a result, the authors successfully obtained a monoclonal antibody (mAb) specific to the sperm membrane protein and showed that the antigen protein interacts with the VE component gp37, a mammalian ZP1 homologue in *X. laevis*, as shown by Far Western blotting. Although the nature of the antigen protein remains to be uncovered, it appeared to be involved in the sperm-VE binding in the fertilization process of *X. laevis* (Kubo *et al*., 2010).

The aim of the present study was to determine which proteins in the sperm membrane components play a role in fertilization of Japanese quail. To achieve this goal, we produced an antibody library against quail sperm membrane components and

tested the potency of the library to inhibit hole formation in the pvm by sperm *in vitro*. In this paper, we provide the first evidence that sperm acrosin is involved in the sperm-pvm binding in Japanese quail.

## Results

### *Effects of monoclonal antibodies on hole formation by sperm on the pvm*

To obtain an antibody that inhibits fertilization from the antibody library, we tested the potency of each culture supernatant forming the library to block hole formation by sperm on the pvm using an *in vitro* assay. Of the culture supernatants of the library, as the supernatant 19A was found to block hole formation strongly (data not shown), so we subjected the 19A cells to cloning and obtained a hybridoma clone (IgG1 isotype) producing mAb 19A16A13. As shown in Figure 1, this mAb efficiently blocked hole formation by sperm (panel B) when it was compared with the control (panel A). The purified antibody from the culture supernatant 19A16A13 definitely inhibited hole formation by sperm in a dose-dependent manner (panel C), and the Fab fragment prepared from the purified mAb also significantly blocked the sperm perforation (panel D), indicating that the inhibitory effect of the mAb was not due to a stereophonic hindrance by binding of the mAb, but rather to the direct binding of the mAb to the molecule indispensable for hole formation. These results strongly suggest that the molecule recognized by the mAb 19A16A13 functions in sperm-egg interaction in quail fertilization.

### *Western blot analysis of the antigen of mAb 19A16A13*

To investigate the nature of the antigen of mAb 19A16A13, we analyzed sperm plasma membrane lysate (SPML) by Western blotting. As shown in Figure 2, the mAb 19A16A13 recognized a 45-kDa protein under non-reducing conditions (lane 1 – in panel A). No band was detected when the blot was incubated with nonspecific

mouse IgG (lane 2 -). The reactivity of the mAb disappeared when SPML was separated by SDS-PAGE under reducing conditions (lane 1 +), indicating that the epitope in the molecule includes disulfide bonds. In addition, since the immunoreactivity of the 45-kDa protein to the mAb was completely lost when SPML was oxidized with periodate after separation by SDS-PAGE and electrotransfer to a polyvinylidene difluoride (PVDF) membrane (panel B, lane +), we concluded that the 45-kDa protein is a glycoprotein whose sugar moiety is included in the epitope of the mAb 19A16A13. This result indicated that the mAb 19A16A13 reacted with a sugar moiety of the glycoprotein.

***Localization of the antigenic 45-kDa protein in sperm***

To analyze the localization of the antigen of the mAb 19A16A13, 45-kDa protein, we processed ejaculated sperm for immunocytochemical observation (Fig. 3). Immunoreactivity was observed in the anterior head of the sperm (panels A and C). No such signal was seen when nonspecific mouse IgG was used as the primary antibody (panel D). To analyze the localization of the antigen more precisely, we performed immunoelectron microscopy for detection of the antigen on the ultra-thin sections of sperm (Fig. 4). In accordance with the results of the immunocytochemical observations, the immunogold particles were distributed in the acrosomal region of the sperm head (panel B). More importantly, the immunoreactive antigen was found within the acrosome (arrows in panel B) and on the surface of the plasma membrane of the acrosomal region (arrowheads in panel B). No such accumulation of gold particles was observed when the specimens were incubated with control IgG (panel A). These observations demonstrated that the antigen reactive to the mAb 19A16A13 specifically localizes in the acrosome region of the sperm head and that this antigen localizes both in the acrosomal matrix and on the surface of the plasma membrane of the sperm head.

***Identification of the antigenic 45-kDa protein by tandem mass spectrometry (MS/MS)***

***analysis***

To elucidate the nature of the antigen of the mAb 19A16A13, 45-kDa protein, we separated SPML with two-dimensional SDS-PAGE, and used MS/MS to examine the tryptic fragments of the immunoreactive spot migrating around 45 kDa in molecular weight. Western blot analysis of the proteins separated by two-dimensional SDS-PAGE revealed 45-kDa and 40-kDa spots (arrowheads in panel B). By laying the X-lay film on the PVDF membrane stained with Coomassie brilliant blue (CBB) after the chemiluminescent detection, we successfully identified the 45-kDa immunoreactive spots on the CBB stained PVDF membrane, and we excised this spot from CBB stained gel for MS/MS analysis of the antigen (arrowhead in panel A). Using MASCOT MS/MS Ions Search, we identified the antigen protein as quail acrosin (GenBank Accession number ABQ40000) with a score of 261, and 7 peptides corresponding to a sequence coverage of 25% were matched to the amino acid sequence of quail acrosin (Table 1). MS and MS/MS data were also analyzed by the *de novo* sequencing and protein identification software PEAKS STUDIO and the sequence tag search tool SPIDER to eliminate false positive results and to improve the sequence coverage. The antigen protein was identified as quail acrosin with a high score, and 7 and 6 peptides were matched to the amino acid sequence of acrosin by PEAKS STUDIO and SPIDER, respectively (Table 1). Results from all search engines used for protein identification were consistent and showed that 9 peptides were matched to quail acrosin with a sequence coverage of 31% in all (Table 1 and Figure 5C), demonstrating that the antigen reactive to the mAb 19A16A13 is quail acrosin.

***Effects of mAb 19A16A13 on the proteolytic activity of 45-kDa sperm acrosin***

To examine whether the mAb 19A16A13 can interfere with the proteolytic activity of sperm acrosin, we performed zymography of SPML, as described in Materials and Methods. As shown in Figure 6, the digested protein bands migrating around 60, 45

and 31 kDa showed the proteolytic activity in the control gel strip, which was incubated in the buffer containing 20 μg/ml mouse IgG (lane 1). When the gel strip was incubated with soybean trypsin inhibitor (SBTI) (lane 5) or leupeptin (lane 6), known to inhibit the enzymatic activity of sperm acrosin, halo formation in all the bands was inhibited, whereas phenylmethylsulfonyl fluoride (PMSF) (lane 4) and bestatin (lane 7) had no effect. Unexpectedly, the incubation of the gel strip with the mAb (lane 2) as well as the Fab fragment of mAb 19A16A13 (lane 3) did not interfere with halo formation. Therefore, the mAb 19A16A13 was found not to inhibit the proteolytic activity of 45-kDa sperm acrosin itself.

***Interaction of sperm acrosin with the pvm***

To characterize the function of the quail sperm acrosin in fertilization, we tested whether the acrosin in SPML interacts with pvm components based on the dot blot assay (Kubo *et al.*, 2010). As shown in Figure 7, the mAb 19A16A13 recognized immunoreactive acrosin that interacts with the pvm component immobilized on a PVDF membrane. When the pvm and /or SPML were omitted from the assay, the immunoreactive signal was diminished to a background level or lower, indicating the specificity and the reliability of the assay performed here. Next, we tested whether the mAb 19A16A13 was able to block the sperm-egg binding directly. We incubated ejaculated sperm with pvm in the presence of pertussis toxin, which inhibits sperm acrosome reaction in quail sperm (Sasanami *et al*., 2007). We found numerous sperm attached to the surface of the pvm after incubation in the absence of the mAb (Fig. 8A), but the attachment was effectively inhibited when the mAb was added to the reaction mixture (Fig. 8B). These results indicated that the 45-kDa sperm acrosin in the plasma membrane of ejaculated sperm supports the binding of sperm to the pvm in quail fertilization.

## Discussion

In the present study, we produced an antibody library against quail sperm membrane components, and the mAb 19A16A13, which strongly inhibits sperm perforation of the pvm *in vitro,* was obtained. From the data of the MS/MS analysis, we showed that quail acrosin to be the antigen reactive to the mAb. To our knowledge, this is the first direct evidence showing that the sperm acrosin plays an essential role in avian fertilization.

For the penetration of pvm, it is assumed that sperm have to bind to the pvm and undergo an acrosome reaction, digest the pvm protein, and penetrate it. Because the mAb, which recognizes the 45-kDa quail acrosin, can inhibit hole formation in the pvm (Fig. 1), the 45-kDa acrosin has a pivotal role in the fertilization. In chickens and turkeys, that the extracts prepared from the ejaculated sperm contain amidase activity based on the potency to degrade gelatin as well as N-$\alpha$-benzoyl-DL-arginine-*p*-nitroanilide as a substrate (Brown & Hartree, 1976; Froman, 1990; Ho & Meizel, 1975; Richardson *et al.,* 1988; Richardson *et al.,* 1992). This sperm amidase in the extracts is considered to be a trypsin-like protease, since the enzyme reaction was inhibited by aprotinin, SBTI, and benzamidine, which inhibit the proteolytic activity of trypsin and the related proteolytic enzymes (Richardson *et al.,* 1992). Quite recently, this amidase was isolated from turkey sperm by gel filtration and directly identified as acrosin by N-terminal Edman sequencing (Slowinska *et al.,* 2010). These reports suggest that the sperm acrosin functions as a lytic agent in the process of sperm penetration by hydrolysis of the pvm in fertilization, although the specific substrate in the pvm has not yet been identified.

In our results, the 45-kDa acrosin showed protease activity that was inhibited by SBTI and leupeptin; however, the hydrolysis of gelatin was not affected by the addition of the mAb 19A16A13 or its Fab fragment (Fig. 6). We did not deny the involvement

of sperm acrosin in the process of pvm decomposition; because the epitope of the mAb includes the sugar moiety of the sperm acrosin by periodate oxidation (Fig. 2B). In mice, the active site of acrosin is a catalytic triad of His, Asp and Ser located in the heavy chain in the molecule (Honda *et al.,* 2002), and the contribution of the sugar moiety to the enzymatic activity has not been demonstrated. Considering these observations along with our results, we suppose that the active site and substrate binding site of quail acrosin are not blocked by the mAb 19A16A13.

More importantly, our results demonstrated that the 45-kDa sperm acrosin interacts with pvm components immobilized on a PVDF membrane (Fig. 7). In addition, the mAb has potency to directly inhibit sperm binding to the pvm (Fig. 8). From the evidence demonstrated here, the 45-kDa acrosin is suggested to mediate the sperm-pvm binding in quail fertilization. It mice, ZP2 binds to proacrosin-null sperm considerably less effectively than wild-type sperm, and the binding of proacrosin to ZP2 is mediated by a strong ionic interaction between polysulphate groups on ZP2 and basic residues on an internal proacrosin peptide (Howes *et al.*, 2001), resulting to conclude that the ZP2-proacrosin interaction is important for the retention of acrosome reacted sperm on the zona pellucida surface. In case of ascidian sperm, paired basic amino acid residues of acrosin are reported to play a key role in the binding of acrosin to the vitelline coat (Kodama *et al.,* 2001). Because ascidian acrosin is released from sperm into the surrounding seawater, acrosin is suggested to be also involved in the process of sperm penetration through the vitelline coat (Kodama *et al.,* 2001).

In our result, since the 45-kDa acrosin localized both on the surface of the sperm head as well as in the acrosomal matrix, based on the immunoelectron microscopic observations (Fig. 4), the 45-kDa acrosin is suggested to be involved in the primary binding of acrosome-intact sperm to the pvm in addition to the hydrolysis of the pvm. We were not able to identify the binding partner of the 45-kDa acrosin in the pvm,

because we failed to detect the specific binding signal of the sperm acrosin to the pvm lysate by Far Western blot analysis (data not shown). Although we did not pursue the discrepancy of the results between the dot blot assay and Far Western blotting, we assume that the interaction of the 45-kDa acrosin with the pvm is not simply mediated via a single molecule but supported by a complex of pvm proteins. Actually, we previously reported that the interaction of ZP1 and ZP3 (Ohtsuki *et al*., 2004; Sasanami *et al*., 2005) as well as that of ZP2 and ZP3 (Kinoshita *et al*., 2010) play a role in the formation of the pvm during follicular development in quail. Moreover, we found that the sperm acrosin contains disufide-bonded three-dimensional arrangement with a modification of sugar moiety in the molecule, though a role of these structures for the sperm-egg interaction in fertilization remains to be studied (Fig. 2). Further experiments will be needed to elucidate the binding machinery of the acrosin and the pvm in fertilization.

Baba *et al*. (1994) demonstrated that acrosin-null male mice produced normal sperm in motility and were fertile; therefore, acrosin is not essential for fertilization, at least in the mouse. Although we are not able to draw a conclusion about whether the sperm acrosin is essential for quail fertilization due to the limitation of the technology (*i.e.*, lack of the gene knockout technique in birds), Adham *et al.* (1997) reported that in an *in vitro* fertilization assay with equal numbers of acrosin-knockout (-/-) and wild type (+/+) sperm present in the medium, all the embryos derived from the fertilized eggs were of the (+/+) genotype. Their finding might indicate the presence of unknown mechanisms for sperm competition related to the function of acrosin in the sperm-egg recognition process. Thus, sperm acrosin is not essential, but plays critical role for fertilization in mice. Actually, We know at present that acrosin is responsible for the dispersal of the acrosomal contents during acrosome reaction (Yamagata *et al.,* 1998). Our current findings in Japanese quail also suggest the importance of sperm acrosin for

fertilization since this amidase is responsible for the process of sperm-egg binding.

In conclusion, this investigation provides the first evidence that the sperm acrosin is responsible for the binding of sperm to the pvm in quail fertilization. Further studies are required to elucidate whether the sperm acrosin is involved in other events of fertilization such as the induction of acrosome reaction, penetration of the pvm or the membrane fusion of gametes.

## Materials and Methods

### *Animals and tissue preparation*

Male and female Japanese quail, *Coturnix japonica*, 15-30 weeks of age (Kato-farm, Toyohashi, Japan), were maintained individually under a photoperiod of 14L: 10D (with the light on at 0500) and were provided with water and a commercial diet (Tokai-Hokuriku Nosan, Chita, Japan) *ad libitum*. The female animals were decapitated and the largest preovulatory follicles were dissected. The granulosa layer from the largest preovulatory follicles was isolated as a sheet of granulosa cells sandwiched between the pvm and basal laminae, as previously described (Gilbert e*t al.* 1977). The pvm was isolated according to a procedure described by Sasanami *et al*. (2002). The pvm was then dissolved in 1% SDS (w/v) buffered at pH 6.8 with 70 m mol $l^{-1}$ Tris–HCl overnight at room temperature. After centrifugation at 10, 000 *g* for 10 min, the supernatants were served as pvm lysates and the protein concentration of the lysates was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). A proctodeal gland secretion was obtained manually from male quail as meringue-like foam. This foam was then centrifuged at 10, 000 *g* for 10 min, and the supernatants collected were stored as proctodeal gland secretion at -80 °C until use.

All the experimental procedures for the use and the care of animals in the present study were approved by the Animal Care Committee of Shizuoka University (approval

number, 22-12).

***Semen collection and preparation***

Ejaculated semen was obtained from male quail prior to mating according to the procedure of Kuroki & Mori (1997). Semen obtained from two to three males was suspended in Hanks' balanced salt solution (HBSS) containing 1.25 m mol $l^{-1}$ of $CaCl_2$ and 0.1% (v/v) of proctodeal gland secretion. We added proctodeal gland secretion to the incubation mixture since it inhibits an agglutination of quail sperm. The concentrations of sperm were measured with a hemocytometer and the sperm viabilities were determined using the LIVE/DEAD sperm viability kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). In all the experiments, sperm were incubated at 39 °C.

***Production of monoclonal antibody***

Ejaculated sperm were washed 3 times with PBS by repeated centrifugation at 800 x *g* for 5 min, and the sperm were then suspended in homogenization buffer containing 110 m mol $l^{-1}$ NaCl, 1 m mol $l^{-1}$ EDTA, 1 m mol $l^{-1}$PMSF, and 50 m mol $l^{-1}$Tris-HCl (pH 7.4) for disruption with an ultrasonic disruptor at the medium power for 10 sec on ice. The sonication was performed for 10 times. The homogenates were centrifuged at 10, 000 x *g* for 10 min for the removal of cellular debris, and the supernatant was ultracentrifuged at 100,000 x *g* for 1 hr at 4°C. The plasma membrane fraction obtained as a precipitate was dissolved in 0.8 ml of lysis buffer (homogenization buffer supplemented with 250 μ mol $l^{-1}$ digitonin and 1 % (w/v) Nonidet P-40) and sonicated as described above. After centrifugation at 20, 000 x *g* for 10 min, the supernatant, referred to as sperm plasma membrane lysate (SPML), was divided into aliquots and stored at -80°C until use.

Immunization of mice with sperm plasma membrane, cell fusion of lymphocytes from immunized mice with myeloma PAI, and establishment of an antibody library

against sperm plasma membrane components were carried out as described previously (Nagai *et al*., 2009).

The mAb 19A16A13-producing hybridoma cloned by screening as described in the next section (*in vitro* assay for sperm-egg interaction) was cultured in 300 ml of HAT supplemented medium, and the mAb 19A16A13 (IgG1 isotype) was purified from the conditioned medium with a Protein A-coupled Affi-gel (Bio-Rad Laboratories) column according to the manufacturer's protocol. Fab fragments of the purified mAb were prepared with a Pierce Fab preparation kit (Pierce, Rockford, IL, USA)

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

To observe the inhibitory activity of each culture supernatant against the sperm penetration of the pvm, a piece of pvm, approximately 8 mm in diameter, was incubated in a micro test tube with 0.5ml of sperm suspension at 1 x $10^7$ sperm /ml in HBSS at 39 °C for 30 min in the presence or absence of the culture supernatant. After 30 min of incubation, the reaction was terminated by placing the tube on ice, and the pvm was washed 3 times with ice-cold PBS. The pvm was transferred onto a glass slide and stained with Schiff's reagent after fixation with 3.7 % (v/v) formaldehyde in PBS. The number of holes formed on the pvm in the x40 field was counted under a light microscope (BX 51, Olympus Optics, Tokyo, Japan). At least five areas were randomly selected for enumeration of perforations.

For the observation of the sperm binding to the pvm, 2 μg/ml pertussis toxin was added to the reaction mixture during the sperm-pvm incubation, which was previously reported to inhibit sperm acrosome reaction in quail (Sasanami *et al.*, 2007). After the incubation, the nuclei of the adherent sperm were stained with 4', 6-diamidino-2-phenylindole (DAPI) after fixation, and the numbers of sperm attached to the pvm were observed under a fluorescent microscope (BX51).

***Gel electrophoresis and Western blot analysis***

SDS-PAGE under non-reducing or reducing conditions was carried out according to Laemmli (1970) using 12% and 5% (w/v) polyacrylamide gel for resolving and stacking, respectively. For Western blotting, proteins separated by SDS-PAGE were electrotransferred to a PVDF membrane (Immobilon-P, Millipore Bedford, MA, USA) (Matsudaira, 1987). The membrane incubated with mAb 19A16A13 (10 μg/ml) followed by horseradish peroxidase-conjugated anti-mouse IgG (Cappel, Durham, NC, USA) as a secondary antibody was visualized by means of a chemiluminescent technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

For periodate oxidation of SPML proteins, a PVDF strip electrotransferred with SPML was oxidized for 20 min with 10 m mol $l^{-1}$ sodium metaperiodate in 100 m mol $l^{-1}$ acetate buffer (pH 5.5) in the dark. After brief rinsing with PBS, the aldehyde group formed was reduced with 100 m mol $l^{-1}$ sodium borohydride in PBS for 15 min. After washing with PBS, the strip was subjected to immunoblotting as described above.

***Zymography***

SPML (10 μg protein) was separated with an SDS-PAGE gel containing 0.1 % (w/v) gelatin according to the procedure described in the previous report (Heussen & Dowdle, 1980). After the electrophoresis, the gel strips were excised along the lane casting, and each gel was incubated with 0.1 mol $l^{-1}$ glycine buffer (pH 8.0) containing 20 μg/ml mouse IgG1, 20 μg/ml mAb 19A16A13, 40 μg/ml Fab fragment of mAb 19A16A13, 1 m mol $l^{-1}$ PMSF, 50 μg/ml soybean trypsin inhibitor (SBTI), 0.5 μg/ml leupeptin, or 40 μg/ml bestatin at 4 °C for 1 hr with gentle agitation. After the incubation, the gel strips were incubated at 37 °C for 3 hr to promote the enzyme reaction. After the reaction, gel strips were stained with CBB, and the halo formation in the gels was observed.

***MS/MS analysis***

SPML (40 μg protein) was separated by SDS-PAGE as described above and the proteins were visualized by CBB staining. The gel strip was excised along with the lane casting and incubated with Laemmli's sample buffer (Laemmli, 1970) without 2-mercaptoethanol for 15 min. After the incubation, the gel strip was loaded on an SDS-PAGE gel, and the proteins were again separated with SDS-PAGE as described above. After the SDS-PAGE, the proteins were electotransferred to a PVDF membrane, and the immunoreactive spot with mAb 19A16A13 was detected as described above. After the detection, the PVDF membrane was washed 3 times with PBS and stained with CBB. After the staining, the X-lay film already developed to have the chemiluminescent signal was laid on the stained membrane, allowing us to identify the antigen spot. The gel strip containing SPML (40 μg) was separated with another SDS-PAGE gel, stained with CBB and a piece of the gel (approximately 1 mm x 1 mm square) containing antigen protein identified as described above was excised. The proteins in the gel were reduced with 10 mM DTT in 50 mM ammonium bicarbonate, S-alkylated cysteine with 55 mM iodoacetamide in 50 mM ammonium bicarbonate and digested with 10 ng/μL of sequence grade trypsin (Promega Corporation, Madison, WI, USA) at 37ºC overnight. The peptides were extracted from the gel with 50% (v/v) acetonitrile (ACN) and 5% (v/v) formic acid, and concentrated using a centrifugal evaporator. The peptide solution was diluted with 10 μL of 0.3% (v/v) formic acid to be suitable for LC-MS/MS analysis.

LC-MS/MS analysis was performed using a linear ion trap time-of-flight mass spectrometer (LIT-TOF MS), NanoFrontier eLD (Hitachi High-Technologies Corporation, Tokyo, Japan) coupled to a nano-flow HPLC, NanoFrontier nLC (Hitachi High-Technologies Corporation). Peptides extracted from the gel were trapped and desalted with a C18 monolith trap column (0.05 mm ID x 150 mm long) (Hitachi High-Technologies Corporation) and then loaded onto a MonoCap C18 Fast-flow

column (0.05 mm ID x 150 mm long) (GL Sciences, Inc.) and eluted with a linear gradient from 2% to 40% solvent B in 60 min at a flow rate of 200 nL/min. Solvent A was 2% ACN and 0.1% formic acid, and solvent B was 98% ACN and 0.1% formic acid. The eluent was ionized with a nano-electrospray ionization source equipped with an uncoated SilicaTip (New Objective, Woburn, MA, USA) and analyzed with a LIT-TOF MS. Mass spectra were obtained in positive ion mode at scan mass range m/z 200–2,000. MS/MS spectra were generated by collision-induced dissociation in the linear ion trap.

To identify the antigen protein, we converted the MS and MS/MS data to a MGF file using a NanoFrontier eLD Data Processing software (Hitachi High-Technologies Corporation) and analyzed the data with MASCOT MS/MS Ions Search (Perkins et al., 1999) (http://www.matrixscience.com/) using the following parameters: Database, NCBInr; Enzyme, Trypsin; Missed Cleavages, 3; Taxonomy, all entries; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (HW) and Oxidation (M); Peptide tolerance, 0.2 Da; MS/MS tolerance, 0.2 Da; Peptide charge, 1+, 2+, and 3+; and Instrument, ESI-TRAP. To confirm the reliability of the result of the protein identification by MASCOT, we also used the *de novo* sequencing and protein identification software PEAKS STUDIO (Bioinformatics Solutions Inc., Waterloo, ON, Canada) (Ma *et al.*, 2003) and the sequence tag search tool SPIDER (Han *et al*., 2005).

***Immunofluorescence microscopy***

Ejaculated sperm were diluted to 1x $10^7$ /ml and fixed in 3.7% (v/v) formaldehyde in PBS at room temperature for 10 min. The fixed sperm were smeared on poly-L-lysine-coated microscope slides. After air drying, the slides were washed with PBS for 5 min, and the cells were incubated with PBS containing 1% BSA and 10% normal goat serum for 1 h for blocking. The cells were then incubated with mAb

19A16A13 (10 μg/ml) or mouse IgG (10 μg/ml) for 2 h at 4 °C. After washing with PBS, they were incubated with Texas red-conjugated sheep anti-mouse IgG (1:200, Cappel) for 1 h at 4 °C. After washing with PBS they were embedded in glycerol and examined under a fluorescence microscope equipped with an interference-contrast apparatus with a 40 x objective (BX 51, Olympus Optics, Tokyo, Japan).

***Immunoelectron microscopy***

Ejaculated sperm embedded in 3% (w/v) agarose were fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol $ml^{-1}$ cacodylate buffer (pH 7.4) overnight at 4 °C. The specimens were embedded in Lowicryl $K_4M$ resin (Polysciences, Warrington, PA, USA). Thin sections were first treated with mAb 19A16A13 (10 μg/ml) or mouse IgG (10 μg/ml), and then with a gold-conjugated goat antiserum against mouse IgG (1:30) (E-Y Laboratories, San Mateo, CA, USA). They were stained with uranyl acetate and observed with a model H-8000 electron microscope (Hitachi, Tokyo, Japan)

***Dot blot assay***

The dot blot assay was performed according to Kubo *et al.* (2010). A PVDF sheet (Immobilon-P; Millipore) wetted with methanol and then with PBS was set in a dot blotter (Bio-Rad Laboratories), and 100 μl of pvm lysate at 10 μg protein/ml was added to each well. Twenty minutes later, the pvm solution was removed by suction, and the wells were washed by suction with PBS three times. Thereafter, the solution in the well was externally aspirated out for disposal. The wells were blocked for 30 min with blocking reagent N101 (NOF Corporation, Japan). After blocking, the wells were rinsed once each with PBS and then with lysis buffer, and 100 μl of SPML at 50 μg protein/ml was added to each well, and the wells were incubated for 30 min. After the incubation, the wells were washed twice with lysis buffer and then once with PBS and were again blocked with the blocker in the same manner. After wells were washed with PBS, 100 μl of culture supernatant was added to each well and the wells were incubated for 30

min. The wells were washed three times with PBS containing 0.1% (w/v) Tween 20, and the bound antibodies were labeled for 30 min with 50 µl/well of peroxidase-conjugated anti-mouse IgG (GE Healthcare) 5,000-fold diluted with 5% (w/v) skim milk in PBS. The wells were washed three times with 0.1% (w/v) Tween 20 in PBS, and the PVDF sheet removed from the blotter was washed again with vigorous shaking three times and then subjected to a chemiluminescent detection system as described above.

***Data Analysis***

Data in Fig. 1C were analyzed for significant differences using ANOVA, and means were compared using Tukey's Multiple Range test. For Fig. 1D, data were analyzed by the student's *t*-test. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

## Declaration of interest

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


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**Figure legends**

Fig. 1 Effects of mAb on the *in vitro* formation of the holes on the pvm by ejaculated sperm. Ejaculated sperm were incubated with pvm in the presence of the conditioned medium of hybridoma 19A16A13 (panel B) or HAT-supplemented medium alone (panel A) at 39 ℃ for 10 min. The pvm was spread on a glass slide, washed with PBS, stained with Schiff's reagent, and observed under a light microscope. Shown are representative photographs of 10 independent experiments. Scale bar = 100 μm.

(C) Ejaculated sperm were incubated with pvm in the presence of purified mAb 19A16A13 (1, 3 or 10 μg/ml) or mouse IgG (10 μg/ml) at 39 ℃ for 10 min, and the number of holes observed in the x400 field under light microscopy was counted. Data shown are the mean ± SD of 3 experiments. Values with different superscripts are significantly different ($P < 0.01$). (D) A Fab fragment was prepared from purified mAb 19A16A13, and ejaculated sperm were incubated with pvm at 39 ℃ for 10 min in the presence of 50 μg /ml Fab fragment (Fab) or vehicle alone (Cont). The number of holes observed in the x400 field under light microscopy was counted. Data shown are the mean ± SD of 3 experiments. An asterisk indicates a significant difference, $P < 0.01$.

Fig. 2 Western blot analysis of SPML with mAb 19A16A13. (A) SPML (5 μg/lane) was separated by SDS-PAGE under non-reducing (-) or reducing (+) conditions, and the proteins were electrotransferred onto a PVDF membrane. The membrane was probed with mAb 19A16A13 (panel 1, 10 μg/ml) or mouse IgG (panel 2, 10 μg/ml). The arrow indicates the immunoreactive band with mAb 19A16A13.

(B) SPML (10 μg/lane) was separated by SDS-PAGE under non-reducing conditions, and the proteins were transferred onto a PVDF membrane. Intact (-) and periodate-oxidized (+) strips were subjected to incubation with mAb 19A16A13 (10 μg/ml). The arrow indicates the immunoreactive band with mAb 19A16A13.

Fig. 3 Localization of the antigen of mAb 19A16A13 in ejaculated sperm. The ejaculated sperm were fixed with 3.7% (v/v) formalin, and the sperm were spread on glass slides. The specimens were incubated with mAb 19A16A13 (10 μg/ml), and the signals were detected with Texas red-labeled anti mouse IgG (panel A, 1:200 dilution). The nuclei of the sperm visualized with DAPI are shown (panel B). C: A merge image of A, B, and DIC is shown. As a control, the mAb 19A16A13 was replaced with mouse IgG (panel D, 10 μg/ml). Bar=50 μm.

Fig. 4 Immunoelectron micrographs of ejaculated sperm. Ultra-thin sections of quail sperm were incubated with mAb 19A16A13 (panel B, 10 μg/ml) or mouse IgG (panel A, 10 μg/ml). The accumulation of gold particles was frequently found in the sperm acrosome when the sections were incubated with mAb 19A16A13 (arrows and arrowheads in panel B), whereas no accumulation was seen on the sections incubated with mouse IgG (panel A). Note that the immunogold particles were localized both on the surface (arrowheads in panel B) and inside (arrows in panel B) of the acrosome. Shown are results representative of repeated experiments. Bar = 500 nm.

Fig. 5 MS/MS analysis of the antigen of mAb 19A16A13. SPML (40 μg protein) was separated by two-dimensional SDS-PAGE, and the proteins were detected with CBB (A) or Western blotting using mAb 19A16A13 (B). The position of the 45-kDa antigen spot is shown (arrow in panel A). (C) The deduced amino acid sequence of quail acrosin (GenBank Accession number ABQ40000). The peptide fragments detected by MS/MS analysis are shown as bold letters.

Fig. 6 Zymography of SPML. SPML (10 μg protein) was separated with a SDS-PAGE gel containing gelatin, and the gel strips were cut along the lane casting. Each gel strip was then incubated with mouse IgG (lane 1), mAb 19A16A13 (lane 2), Fab fragment of the mAb (lane 3), PMSF (lane 4), SBTI (lane 5), leupeptin (lane 6), or bestatin (lane 7). One additional strip was processed for Western blotting with mAb

19A16A13 (lane 8). The position of the 45-kDa acrosin is shown by an arrow on the right side of the figure. Shown are results representative of three repeated experiments.

Fig. 7 Dot blot assay with mAb 19A16A13. SDS-solubilized pvm or lysis buffer alone was dot blotted onto a PVDF sheet by the aid of a dot blotter and then blocked with N101 blocking reagent. Each dot was incubated with SPML (50 μg/ml) or lysis buffer only as a control to bind the sperm membrane components in SPML. The dots were incubated with mAb 19A16A13 and then with a horseradish peroxidase-coupled secondary antibody. The PVDF sheet was visualized with an ECL detection system. Shown are results representative of three repeated experiments.

Fig. 8 Effects of mAb 19A16A13 on the interaction of ejaculated sperm and pvm. Ejaculated sperm were incubated with pvm in the presence of 20 μg/ml of mAb 19A16A13 (panel B) or mouse IgG (panel A). After washing with PBS, the pvm was spread on glass slides, fixed with formalin and observed under a fluorescence microscope. The sperm nuclei were stained with DAPI. Shown are representative photographs of two independent experiments. Bar = 20 μm.