Serine protease activity, bovine sperm protease, 66 kDa (BSp66), is present in hamster sperm and is involved in sperm–zona interaction

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Abstract

Bovine sperm protease, 66 kDa (BSp66) is a serine protease previously characterized in bovine spermatozoa. Like other proteases, it may be present in sperm from other mammalian species different from bovine, playing a role in the fertilization process. In this study, we looked for BSp66 in hamster spermatozoa using heterologous antibodies against bovine BSp66. An immunoreactive protein was detected by Western blotting in mature and immature sperm. The detected protein had two isoforms similar to the ones reported in bovine sperm. Furthermore, indirect immune detection by fluorescence and electron microscopy assays, showed BSp66 signal at the acrosomal region similar to bovine sperm. As it was determined in bovine sperm, the acrosomal reaction displays the antigen within the acrosomal content. When live hamster sperm was incubated with polyclonal antibody against bovine BSp66 a decrease in the number of sperm bound to zona pellucida in homologous IVF and an impairment of head–head agglutination, were observed. These results suggest that a protease homologous to bovine BSp66 is present in golden hamster spermatozoa, with a conserved molecular mass and cellular location. Moreover, hamster BSp66 is probably involved in zona pellucida recognition.

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Introduction

The presence of several enzymes, such as acrosin and other trypsin-like proteases, had been demonstrated in the mammalian sperm acrosome (Honda *et al.* 2002). Acrosome contains a range of catalytic activities including proteases which compound a highly redundant system (Parrish & Polakoski 1979, Kohno *et al.* 1998, Ohmura *et al.* 1999, Morales *et al.* 2003). Some controversy concerning the function of acrosomal proteases has been discussed previously (Bedford 1988). Proteases are related to the enzymatic lysis of the oocyte investments and to the processing of molecules involved in sperm signal transduction pathways leading to acrosome reaction (AR) (Tesarik 1995, Dell *et al.* 1999, Haden *et al.* 2000, Yoshitani *et al.* 2001, Klinefelter *et al.* 2002). Indeed, various trypsin inhibitors have been reported to effectively block the sperm penetration of the zona pellucida (ZP) *in vitro* (Zaneveld *et al.* 1971, Fraser 1982, Llanos *et al.* 1993). We have already characterized a putative new member of this protease family named bovine sperm protease,

66 kDa (BSp66). BSp66 is a trypsin-like serine protease located at the sperm head of bovine spermatozoa which showed no-crossreactivity with anti-acrosin/pro-acrosin antibodies (Cesari *et al.* 2004a,b). BSp66 dimerizes by disulfide bonds into a higher form named BSp120 (120 kDa) when bovine spermatozoa is cryopreserved (Cesari *et al.* 2003). As reported for the widespread acrosin system, BSp66 is supposed to be present in other mammalian spermatozoa. In this work we investigated the presence of BSp66 in hamster spermatozoa using heterologous antibodies against bovine BSp66 and its role during *in vitro* fertilization.

Materials and Methods

Chemicals and antibodies

Ionophore A23187, 4-aminobenzamidine hydrochloride (pABA), BSA (fraction V), Tween 20, FITC labelled anti-serum against rabbit IgG (monoclonal) and embryo culture reagents used for *in vitro* fertilization were purchased at

Sigma–Aldrich (St Louis, MO, USA). All other reagents were of analytical grade and were obtained from the best commercially available sources. Chemicals for electron microscopy were from Pelco (Redding, CA, USA). Polyclonal antibodies against BSp120 and BSp66 were prepared in our laboratory from partially purified BSp66 as described previously (Cesari *et al.* 2004a). Briefly, the electrophoretic band corresponding to partially purified BSp66 from bovine fresh sperm was cut and mixed with complete Freund's adjuvant. Male rabbits were injected subcutaneously with 50 µg of BSp66 protein. Animals were bled, and the titer of the specific antibodies was determined by Western immunoblotting. Control non immune serum was obtained before immunization.

Sperm isolation

Epididymides were obtained from sexually mature (2–3 months old) *Mesocricetus auratus* (golden hamster). Spermatozoa were dispersed from dissected caput or cauda epididymidis in 1 ml HM medium (8.3 mM Hepes, 36.3 mM NaCl, 1.59 mM KCl, 0.39 mM MgSO₄, 0.33 mg/ml glucose, 0.74% Na-lactate, 0.4 mM KH₂PO₄, 0.11 mg/ml sodium pyruvate, 1.7 mM CaCl₂, pH 7.3; Pietrobon *et al.* 2003), washed in PBS (Sigma P-4417, pH 7.4) and finally suspended in HMB medium to allow capacitation (HM media plus 25 mM NaHCO₃, 3 mg/ml BSA, pH 7.4; Pietrobon *et al.* 2003). Capacitation was promoted at 37°C, 5% CO₂ and saturated humidity (NAPCO 5004) during 90 min. Non capacitating conditions were achieved by incubation in HM medium. After capacitation, AR was induced by the addition of 10 µM A23187 ionophore in DMSO for 15 min. Controls were performed with similar concentration of DMSO. Animals were handled under NIH Animal Care Guide.

Control experiments were performed with bovine spermatozoa. Frozen bovine semen pills were generously provided by INTA (Balcarce, Argentina). Each pill (250 µl) contains 50 × 10⁶ sperm/ml. Cells were preserved in liquid nitrogen (–196°C) until used.

Assessment of sperm motility and viability

Sperm vitality

Sperm vitality was verified by checking motility and eosin vital dye (Eliasson & Treichl 1971). Briefly, wet mounts of spermatozoa were prepared to evaluate cell motility. Estimation of the percentage of progressive motile spermatozoa was performed by microscopy at 400×. Two independent observers scored the results. Samples with less than 80% of motile spermatozoa before and after capacitation were not included in the study. On the other hand, one drop (10 µl) of the sperm suspension was diluted in the same volume of 1% (w/v) eosin in 0.9% (w/v) NaCl. Samples with less than 80% living spermatozoa were not included.

Western blotting

Caput and caudal epididymal sperm, capacitated or non capacitated, were washed twice in PBS and protein extracts were obtained. Sperm suspensions containing 13 × 10⁶ spermatozoa/ml were mixed with 5 × Laemmli sample buffer (40 µl for each 10 × 10⁶ sperm) with or without 25 mM β-mecaptoethanol (mce). Equal amounts of cells were loaded onto each lane (7.5 × 10⁶ sperm). Samples were electrophoresed in 10% (w/v) polyacrylamide gels (SDS-PAGE) at 4°C and run at 20 mA/gel, according to Laemmli (1970). Molecular weight was estimated using protein standards: β-galactosidase (116 kDa), BSA (66 kDa) and ovalbumin (45 kDa). Then, proteins were transferred onto nitrocellulose membranes at 190 mA for 30 min in Trans-Blot SD-cell device (Bio-Rad). The transfer solution contained 48 mM Tris/39 mM glycine (pH 9.2) and 20% (v/v) methanol. The membranes were blocked with 0.2% Tween 20, 5 mg/ml BSA in PBS (blocking buffer) at room temperature for 2 h and incubated with anti-BSp66 (1:200) and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG (1:7500). After extensive washing, the membranes were immersed in alkaline phosphatase buffer (Harlow & Lane 1998), containing 0.33 mg Nitro Blue Tetrazolium and 0.01 mg 5-bromo-4-chloro-3-indolyl phosphate per ml until colour development.

Zymography

Polyacrylamide gel electrophoresis was performed as described for Western blotting. The gel solutions were supplemented with 0.1% (w/v) gelatine (Hummel *et al.* 1996). The gel was washed in 2.5% (v/v) Triton X – 100, 5% (w/v) CaCl₂ and incubated for 20 h at 39°C in 0.1 M Tris–HCl (pH 7.5). Proteolytic activity was visualized as unstained regions after Coomassie Brilliant Blue (CBB) staining.

Immunofluorescence labeling assay (IFLA)

Hamster spermatozoa obtained from caput or distal cauda epididymis were incubated under capacitating or non-capacitating conditions as described above. Cells were washed and suspended in PBS at room temperature. Sperm concentration was adjusted to 50 × 10⁶ spermatozoa/ml. Cells were fixed with 2% formaldehyde in PBS for 10 min, washed and smeared on glass slides covered with 0.1% polylysine. Dried smears were permeabilized in 50% methanol for 5 min, washed and incubated with blocking buffer. Then, cells were incubated with anti-BSp66 or non immune serum diluted 1:20 for 1 h in a humidified chamber. After thoroughly washing, smears were incubated with swine anti-rabbit immunoglobuline fluorescein isothiocyanate (FICT)-conjugated (Sigma–Aldrich) diluted 1:100 for 1 h at 37°C. Then, smears were washed and mounted with PBS:glycerol (1:9). Specimens were examined with a Epi fluorescence Nikon Optiphot II microscope (Nikon Instech Co. Ltd, Kawasaki, Japan) equipped with a 40/1.25 fluorescence planapo chromatic objective (40×).

Immune electron microscopy (IEM)

Golden hamster spermatozoa obtained from caput or distal cauda were incubated under capacitating or non-capacitating conditions. AR was induced as described above. Immune labelling was performed on live spermatozoa from all experimental conditions (i.e. non capacitated, capacitated and acrosome reacted). Cells were separated in equal aliquots and incubated with blocking buffer for 50 min, washed twice and incubated for 1 h on an orbital shaker with anti-BSp66 (1:5), or non immune rabbit serum (1:5) as a negative control. After thoroughly washing ($200 \times g$, 15 min), cells were incubated with the secondary antibody for 1 h on an orbital shaker (1:10 anti-rabbit IgG labelled with 10 nm colloidal gold, Sigma–Aldrich), washed again, and fixed overnight in 2% glutaraldehyde in PAF (2% picric acid, 4% paraformaldehyde in PBS). After washing, samples were post fixed in 4% OsO₄ in PBS for 3 h. Sperm samples were dehydrated, embedded and sectioned for transmission electron microscopy. Thin sections were observed at 50 Kv in a Zeiss EM 900 microscope (Carl Zeiss Argentina S.A. C1430BC0, Buenos Aires).

In vitro fertilization (IVF)

Female hamsters were superovulated by intraperitoneal sequential injection of 20 IU of PMSG (Serono Inc. One Technology Place, Rockland, MA, USA) and hCG (Elea SACIFgA, Sanabria 2353, Buenos Aires, Argentina). Animals were killed 17 h after hCG injection, oviducts were

removed and the swollen ampula were flushed with HEMC-3 medium (110 mM ClNa, 4.7 mM ClK, 1.9 mM Cl₂Ca, 0.29 mM Cl₂Mg, 15 mM CO₃HNa, 0.018% sodium lactate, 29 µg/ml L-Glutamine, 1.9 mM glycine, 0.109 mg/ml hypotaurine, 3 mg/ml BSA V, 65 µg/ml penicillin, 0.5% streptomycin, 11.9 mM Hepes; Ogura & Yanagimachi 1993) to recover the egg–cumulus complexes (ECCs). The cumulus cells were removed in the same medium containing 290 U/ml hyaluronidase (bovine testicular origin, Sigma–Aldrich). Groups of 12 eggs were transferred to HEMC-3 drops until insemination. Spermatozoa from cauda epididymis were washed, concentration adjusted to 5×10^6 sperm/ml and incubated with anti-BSp66 (1:5 or 1:10), non immune serum or media under capacitating conditions as described above during 60 min. Then, spermatozoa were washed by centrifugation ($200 \times g$, 15 min) to eliminate unbound antibodies and used to inseminate oocytes with a final concentration of 0.2×10^6 sperm/ml. After 30 min of co-culture in HEMC-3 medium, oocytes were washed with a micropipette and the number of spermatozoa bound to the ZP were counted under Stemi SU6 Stereomicroscope (Carl Zeiss Argentina S.A. C1430BC0, Buenos Aires) ($20 \times$). The results were analysed by *t*-test.

Results

BSp66 presence in hamster spermatozoa

Two bands of 66 and 116 kDa were detected by the heterologous antibody against bovine BSp66 in cauda

Figure 1 (A) Western blotting of protein extracts from golden hamster and bovine spermatozoa. Protein extracts from bovine or hamster sperm were obtained in the presence or absence of 25 mM β-mecaptoethanol (mce) as indicated in each lane. Lane 1, non-capacitated cryopreserved bovine spermatozoa; lane 2, non-capacitated golden hamster caput spermatozoa; lane 3, non-capacitated golden hamster cauda spermatozoa; lanes 4 and 5, capacitated golden hamster cauda spermatozoa. (B) Gelatine zymography of proteases from sperm extracts (50 µg) under non-reducing conditions. Lane 1, Mr standards; lane 2, cryopreserved bovine spermatozoa; lane 3, hamster spermatozoa from distal cauda. Mr was estimated using protein standards: β-galactosidase (116 kDa), BSA (66 kDa) and ovalbumin (45 kDa) indicated on the left (kDa). The positions corresponding to BSp66 and BSp120 (arrows) and acrosin gelatinolytic band (acr; arrowhead) are indicated.

and caput spermatozoa by Western blotting assays (Fig. 1A), corresponding to both forms of the protease named BSp66 and BSp120, respectively. Only the one corresponding to BSp120 was observed in capacitated

Figure 2 Immunofluorescent staining with polyclonal antibodies against bovine sperm BSp66 (1:20). Golden hamster spermatozoa obtained from caput or cauda epididymis were incubated under capacitating or non-capacitating conditions, fixed, mounted and permeabilized in 50% methanol previous to incubation with antibodies. A and B, Control with non-immune serum (1:20); C and D, caput sperm; E and F, cauda sperm non-capacitated; G and H, cauda sperm-capacitated. Panels B, D, F and H are phase contrast images. Original magnification 400 ×.

spermatozoa in the absence of mce, while both BSp66 and BSp120 were observed under non-capacitating conditions (Fig. 1A, lanes 3 and 4). When capacitated spermatozoa were incubated in the presence of the reducing agent, again both BSp120 and BSp66 were observed (Fig. 1, lanes 4 and 5). These results may be explained by our hypothesis of the physiological dimmerization of BSp66 into BSp120 during capacitation, as the active mature form of the protease.

The activity of the protease in crude sperm extracts was evaluated by zymography under non-reducing conditions (Fig. 1B). The active bands corresponding to the electrophoretic mobility of both BSp66 and BSp120 were observed in non-capacitated hamster spermatozoa obtained from distal cauda and as expected, in cryopreserved bovine sperm extract (Fig. 1B, arrows), demonstrating that this enzyme is active in hamster spermatozoa. Since crude extracts of hamster spermatozoa were used, 39–49 kDa bands corresponding to acrosin reported by Polakosky & Zaneveld (1976) were also detected (Fig. 1B, arrowhead).

Location of BSp66 in sperm from cauda and caput epididymis

When location was assessed by IFLA, the fluorescent signal was observed on the acrosomal region in fixed/permeabilized capacitated and non-capacitated spermatozoa from cauda and caput epididymis, indicating that the localization of the protein does not change during the epididymal transit nor during capacitation (Fig. 2). The location of the protein homologous to BSp66 in hamster is coincident with that observed in bovine sperm (Cesari *et al.* 2004b). No signal was observed in the negative control (Fig. 2A).

BSp66 was detected at the plasma membrane (PM) over the acrosome (Fig. 3A) in live capacitated spermatozoa by IEM. In acrosome reacting spermatozoa the signal towards BSp66 was observed surrounding the exocytotic vesicles (Fig. 3B) suggesting that BSp66 is located onto the PM or the outer acrosomal membrane (OAM), as described for bovine spermatozoa (Cesari *et al.* 2004b). In some cases, the acrosomal content showed gold labelling (Fig. 3C), suggesting that BSp66 would be within the acrosomal vesicle and also membrane bound. BSp66 signal was specific, as control assays displayed no signal (Fig. 3D). The high concentration of serum used during 1 h may have induced a high degree of sperm agglutination, affecting the binding of the antibodies. In order to control this effect, the assay was performed with spermatozoa that had been fixed before the incubation with primary and secondary antibodies, obtaining identical results (not shown).

Antiserum against BSp66 impairs ZP binding and head–head agglutination

Taking into account that the heterologous antibody recognized a protein with the same molecular mass and location to BSp66, hamster capacitated spermatozoa from

Figure 3 Ultrastructural localization of BSp66 by transmission electron microscopy. Sections of golden hamster spermatozoa obtained from cauda were incubated under capacitating conditions. AR was induced by calcium ionophore A23187 ($10 \mu\text{M}$), cells were blocked, incubated with primary (non-immune serum or anti-BSp66, 1:20) and secondary antibodies (10 nm colloidal gold-labelled anti-rabbit IgG). Then, fixation was performed and sperm samples were dehydrated, embedded and sectioned. A, longitudinal section through capacitated sperm head incubated with anti-BSp66; B and C, longitudinal section through acrosome reacted sperm head incubated with anti-BSp66; D, longitudinal section through the sperm head incubated with non-immune serum (control). Magnification is indicated above the figures.

Table 1 Zona binding of golden hamster sperm incubated with anti-BSp66. *In vitro* fertilization assay was performed as routine in the presence of golden hamster sperm incubated with the mentioned antiserum. The number of sperm bound to zona pellucida (ZP) was determined. Results are averages of four identical assays. Percentages of inhibition refer to total sperm bound to ZP in control treatment. Statistical analysis of the data was performed by T test.

Treatment	No. sperm bound to ZP	S.E.M.	n	Inhibition of binding to ZP (%)
Control	105.6	11.37	9	0 ^a
Non-immune serum	137.0	7.84	11	0 ^a
Anti-BSp66 (1:10)	34.44	2.30	9	74.9 ^b
Anti-BSp66 (1:5)	21.38	2.80	8	84.4 ^b

^{a,b}Different superscripts are statistically different ($P < 0.05$).

Figure 4 *In vitro* fertilization of golden hamster oocytes. Golden hamster spermatozoa obtained from cauda epididymis were capacitated and incubated with HMB medium (A), non-immune serum (control) (B), anti-BSp66 (1:10) (C) or anti-BSp66 (1:5) (D). Sperm were washed to remove non-bound antibodies and co-incubated with oocytes. After 20 min oocytes were washed and spermatozoa bound to ZP were observed. This result is representative of four experiments. Magnification 40 ×.

cauda were incubated with increasing doses of anti-BSp66 (1:5 and 1:10), in order to investigate the function of hamster BSp66 in the fertilization process. Sperm incubation with non-immune serum did not affect sperm motility and viability (not shown) nor the binding to ZP (Table 1, Fig. 4A and B). When spermatozoa were incubated with anti-BSp66 the number of sperm bound to ZP decreased significantly (Table 1, Fig. 4C and D), indicating that BSp66 blocking affects ZP binding or a previous step of fertilization. There was no statistical difference between the doses of antiserum assayed, but an increasing tendency of binding impairment was observed with the higher concentration of antiserum used. Pronuclei were

observed in several oocytes after 1 h of gamete co-incubation (not shown). Spermatozoa not bound to ZP were also analysed (Fig. 5). Agglutination occurred in spermatozoa incubated with non-immune serum, while a complete impairment of agglutination was observed when either 1:5 or 1:10 anti-BSp66 was used, suggesting that the antibody may have blocked some surface ligands.

Discussion

Fertilization involves sperm binding and crossing of the ZP. Both events are thought to occur by mechanical force and/or enzymatic hydrolysis (Yanagimachi 1994, Bedford

Figure 5 Head-head agglutination of capacitated spermatozoa. *In vitro* fertilization was performed as described in Fig. 4. Non-bound spermatozoa were observed during co-incubation and numbers of agglutinates were counted. A, capacitated spermatozoa incubated with non-immune serum (1:10); B, capacitated spermatozoa incubated with anti-BSp66 (1:10). Magnification 40 ×.

1998). A number of acrosomal serine proteases other than acrosin are present in various mammalian sperm. This serine protease system varies among the different species on the basis of their gelatine-hydrolysing activity on SDS-polyacrylamide gel electrophoresis (Honda *et al.* 2002). We have previously found a putative new serine protease in bovine sperm, named BSp66 for bovine sperm protease of 66 kDa (Cesari *et al.* 2003), located at the sperm head surface. This protease was partially purified from bovine sperm and it was demonstrated that it showed no cross-reactivity with anti-acrosin/pro-acrosin antibodies (Cesari *et al.* 2004a). In the present report, we demonstrate that a protein that cross-reacts with heterologous anti-BSp66 is also present and enzymatically active in hamster spermatozoa. This BSp66 is conserved in molecular mass and location at the acrosomal region of hamster spermatozoa. In our previous work, two forms were identified as a monomer and a dimer of a unique protein that formed disulfide bonds after membrane modification induced by cryopreservation or cold storage (Cesari *et al.* 2003). We reported that the dimeric form, named BSp120 was only found in cryopreserved bovine spermatozoa, while the monomeric form, BSp66, was present in fresh bovine sperm samples. The presence of the two forms of BSp in fresh hamster spermatozoa may be due to cold dimerization, since sperm extracts were stored at -20°C for several days before the zymography assay. Since 'cryocapacitation' is a known process (Cormier & Bailey 2003), we have speculated that this dimerization might occur during physiological capacitation. In the present work the two forms, 66 and 120 kDa, were also observed in protein extracts from non-capacitated hamster epididymal spermatozoa, but only the dimer was detected in capacitated spermatozoa. This observation suggests that a capacitation-induced dimerization would be possible. The capacitation period involves surface changes in several species (Visconti *et al.* 1999), some of them associated with the unmasking of surface receptors such as the progesterone receptor (Sirivaidyapong *et al.* 2001, Pietrobon *et al.* 2003). In agreement with these findings, our previous results with live bovine spermatozoa showed that capacitation induction promoted the exposure of BSp66 onto the sperm surface, and a redistribution during the AR (Cesari *et al.* 2004b).

As BSp66 codifying genes were not yet identified, we ignored this protein if it was expressed in the spermatogonial cell or if it was superficially acquired during the journey across the male duct (Boue *et al.* 1992). In this report, BSp66 was detected on the acrosomal region of hamster sperm recovered along the epididymis, suggesting that the protein may be expressed early in male germ cells. The exposure of BSp66 during capacitation was not evaluated because the assays were performed on permeabilized hamster spermatozoa. AR induction depicted a different exposure of BSp66 in hamster live spermatozoa, showing that BSp66 was located not only over the external membranes but also within the acrosomal content, as described

for acrosin (Honda *et al.* 2002). These results are consistent with our previous findings in bovine spermatozoa (Cesari *et al.* 2004b).

In this report, antibodies against bovine BSp66 significantly reduced the number of spermatozoa bound to hamster ZP, although a complete impairment of zona binding was not observed. The fact that blocking antibodies against sperm surface antigens did not completely impair fertilization was also observed in our previous assays with bovine sperm (Cesari *et al.* 2004b) and by other authors (Ramalho-Santos *et al.* 2000). Although the physiological interpretation of IVF results in the presence of antibodies against specific proteins is controversial, the role of sperm and oocyte surface proteins has been determined by this experimental approach (Hasegawa *et al.* 2000, Peknicova *et al.* 2001). Surprisingly, oocytes with two pronuclei were detected after 1 h of co-incubation, suggesting that anti-BSp66 did not block sperm-egg fusion or other events posterior to sperm entry into the oocyte. Primary or secondary zona binding may be the susceptible events.

In several mammalian species head-head agglutination of spermatozoa has been observed when epididymal sperm is diluted or when incubated in presence of serum (Harayama *et al.* 2000, Grace *et al.* 2002). A complete impairment of head-head agglutination was observed when spermatozoa were incubated with anti-BSp66 antiserum suggesting that BSp66 may be a surface ligand. This observation is consistent with the hypothesis supported by other authors, that the role of serine proteases in the ZP lysis may be indirect; i.e. the inhibition of the sperm-ZP interaction by serine protease inhibitors may be a consequence of a deficient capacitation or AR (Honda *et al.* 2002, Morales *et al.* 2003). On the basis of this hypothesis, their role seems to be the processing of other proteins in the acrosome or on the membranes during the AR, probably as part as the signal transduction pathways as it was demonstrated for the human sperm proteasome (Morales *et al.* 2003). Thus, whether BSp66 is a membrane protease and its roles during AR or gamete interactions are still under investigation.

Immunological cross-reaction shows a relation between bovine and hamster BSp66, however, protein sequencing is needed to confirm that it is a conserved protein. We are pursuing to purify to homogeneity, sequence and clone the immunoreactive protease in order to provide substantial evidence about the novelty of this protease and of how widespread it is.

In summary, we conclude that a protein homologue to bovine BSp66 is present in golden hamster spermatozoa with a conserved molecular mass and location at the acrosomal region. Moreover, hamster BSp66 is involved in early steps of ZP recognition.

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References

- Bedford JM 1998 Mammalian fertilization misread? Sperm penetration of the eutherian ZP is unlikely to be a lytic event. *Biology of Reproduction* **59** 1275–1287.
- Boue F, Lassalle B, Duquenne C, Villaroya S, Testart J, Lefevre A & Finaz C 1992 Human sperm proteins from testicular and epididymal origin that participate in fertilization: modulation of sperm binding to zona-free hamster oocytes, using monoclonal antibodies. *Molecular Reproduction and Development* **33** 470–480.
- Cesari A, Cacciato CS, De Castro RE & Sánchez JJ 2003 Low temperature-induced dimerization of the bovine sperm serine protease, BSp66. *Journal of Cellular Biochemistry* **88** 1057–1065.
- Cesari A, Cacciato CS, de Castro R & Sánchez JJ 2004a Partial purification and characterization of a trypsin-like serine protease from bovine sperm. *International Journal of Andrology* **27**(5) 311–318.
- Cesari A, Sánchez JJ, Biancotti JC, Vazquez-Levin MH, Kaiser G, Palma G, Alberio R, Vincenti A & Fornes MW 2004b Immunolocalization of bovine sperm protease BSp120 by light and electron microscopy during capacitation and the acrosome reaction. Its role in *in vitro* fertilization. *Molecular Reproduction and Development* **69**(4) 411–418.
- Cormier N & Bailey JL 2003 A differential mechanism is involved during heparin- and cryopreservation-induced capacitation of bovine spermatozoa. *Biology of Reproduction* **69** 177–185.
- Dell A, Morris HR, Easton RL, Patankar M & Clark GF 1999 The glycobiology of gametes and fertilization. *Biochimica et Biophysica* **1473** 196–205.
- Eliasson R & Treichl L 1971 'Supravital staining of human spermatozoa'. *Fertility and Sterility* **22** 134–137.
- Fraser LR 1982 p-Aminobenzamidine, an acrosin inhibitor, inhibits mouse sperm penetration of the zona pellucida but not the acrosome reaction. *Journal of Reproduction and Fertility* **65** 185–194.
- Grace KS, Bronson RA & Ghebrehwet B 2002 Surface expression of complement receptor gC1q-R/p33 is increased on the plasma membrane of human spermatozoa after capacitation. *Biology of Reproduction* **66** 823–829.
- Haden NP, Hickox JR, Whisnant CS & Hardy DM 2000 Systematic characterization of sperm-specific membrane proteins in swine. *Biology of Reproduction* **63** 1839–1847.
- Harayama H, Miyake M & Kato S 2000 Role of cyclic adenosine 3',5'-monophosphate and serum albumin in head-to-head agglutination of boar spermatozoa. *Reproduction, Fertility and Development* **307**–318.
- Harlow E & Lane D 1998 Antibody Response. In *Antibodies: A laboratory manual*. pp55–120. Eds Marlow E & Lane E. New York: Cold Spring Harbour, Cold Spring Harbor Laboratory.
- Hasegawa A, Tsubamoto H, Hamada Y & Koyama K 2000 Blocking Effect of Antisera to Recombinant zona pellucida Proteins (r-ZPA) on *In Vitro* Fertilization. *American Journal of Reproductive Immunology* **44** 59–64.
- Honda A, Siruntawineti J & Baba T 2002 Role of acrosomal matrix proteases in sperm–zona pellucida interactions. *Human Reproduction Update* **8** 405–412 (Review).
- Humnet KM, Perheiter AR, Guthman AC & Litty WW 1996 Anomalous estimation of protease molecular weight using gelatin-containing SOS–PAGE. *Analytical Biochemistry* **233**(1) 140–142.
- Klinefelter GR, Welch JE, Perrault SD, Moore HD, Zucker RM, Suarez JD, Roberts NL, Bobseine K & Jeffay S 2002 Localization of the sperm protein SP22 and inhibition of fertility *in vivo* and *in vitro*. *Journal of Andrology* **23** 48–63.
- Kohn N, Yamagata K, Yamada S, Kashiwabara S, Sakai Y & Baba T 1998 Two novel testicular serine proteases, TESP 1 and TESP 2 are present in the mouse sperm acrosome. *Biochemical and Biophysical Research Communications* **245** 658–665.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227** 680–685.
- Llanos M, Vigil P, Salgado AM & Morales P 1993 Inhibition of the acrosome reaction by trypsin inhibitors and prevention of penetration of spermatozoa through the human zona pellucida. *Journal of Reproduction and Fertility* **97** 173–178.
- Morales P, Kong M, Pizarro E & Patsen C 2003 Participation of the sperm proteasome in human fertilization. *Human Reproduction* **18** 1010–1017.
- Ogura A & Yanagimachi R 1993 Round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy. *Biology of Reproduction* **48** 219–225.
- Ohmura K, Kohn N, Kobayashi Y, Yamagata K, Sato S, Kashiwabara S & Baba T 1999 A homologue of pancreatic trypsin is localized in the acrosome of mammalian sperm and is released during acrosome reaction. *Journal of Biological Chemistry* **274** 29426–29432.
- Parrish RF & Polakoski KL 1979 Mammalian sperm proacrosin-acrosin system. *International Journal of Biochemistry* **10** 391–395.
- Peknicova J, Capkova J, Geussova G, Ivanova M & Mollova M 2001 Monoclonal antibodies to intra-acrosomal proteins inhibit gamete binding *in vitro*. *Theriogenology* **56** 211–223.
- Pietrobon EO, Monclus MA, Alberdi AJ & Fornés MW 2003 Progesterone receptor availability in mouse spermatozoa during epididymal transit and capacitation. Ligand blot detection of progesterone binding protein. *Journal of Andrology* **24** 612–620.
- Polakoski KL & Zaneveld LJD 1976 Proacrosin. In *Methods in enzymology Proteolytic enzymes Part B*. Ed. Lorand L, pp 123–132. New York: Academic Press.
- Ramallo-Santos J, Moreno RD, Sutovsky P, Wing-Sang Chan A, Hewitson L, Wessel GM, Simerly CR & Schatten G 2000 SNAREs in mammalian sperm possible implications for fertilization. *Developmental Biology* **223** 54–69.
- Sirivaidyapong S, Bevers MM, Gadella BM & Colenbrander B 2001 Induction of the acrosome reaction in dog sperm cells is dependent on epididymal maturation: the generation of a functional progesterone receptor is involved. *Molecular Reproduction and Development* **58** 451–459.
- Tesarik J 1995 The role of proteases in the mammalian sperm acrosome reaction. In *Human sperm acrosome reaction*, pp 123–132. Eds P Fénichel & J Parinaud John Libby Curotext, Mantrouge, Paris.
- Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA & Kopf GS 1999 Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Developmental Biology* **14** 429–443.
- Yanagimachi R 1994 Mammalian fertilisation. In *The Physiology of Reproduction*, pp 189–317. Eds E Knobil & J Neill. New York: Raven Press.
- Yoshitani N, Mori E & Takasaki S 2001 Detection of carbohydrate recognition molecules on the plasma membrane of boar sperm by dextran-based multivalent oligosaccharide probes. *Glycobiology* **11** 313–320.
- Zaneveld LJD, Polakoski KL, Robertson RT & Williams WL 1971 Trypsin inhibitors and fertilization. In *Proceedings of the first international conference on proteinase inhibitors*, pp 236–244, Ed. Walter de Gruyter, Berlin, Germany.

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