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

A Cesari, M R Katunar, M A Monclus\(^1\), A Vincenti\(^1\), J C de Rosas\(^1\) and M W Forné\(^1\)

Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CC: 1245 (7600) Mar del Plata, Buenos Aires, Argentina and \(^1\)Instituto de Histología y Embriología de Mendoza, Área de Histología y Embriología, Departamento de Morfología y Fisiología, Facultad de Ciencias Medicas, Universidad Nacional de Cuyo – CONICET, CC: 56 (5500) Mendoza, Argentina

Correspondence should be addressed to A Cesari; Email: acesari@mdp.edu.ar

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.


Introduction

The presence of several enzymes, such as acrosin and other trypsin-like proteases, had been demonstrated in the mammalian sperm acrosome (Honda \textit{et al.} 2002). Acrosome contains a range of catalytic activities including proteases which compound a highly redundant system (Parrish & Polakoski 1979, Kohno \textit{et al.} 1998, Ohmura \textit{et al.} 1999, Morales \textit{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 \textit{et al.} 1999, Haden \textit{et al.} 2000, Yoshitani \textit{et al.} 2001, Klinefelter \textit{et al.} 2002). Indeed, various trypsin inhibitors have been reported to effectively block the sperm penetration of the zona pellucida (ZP) \textit{in vitro} (Zaneveld \textit{et al.} 1971, Fraser 1982, Llanos \textit{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 \textit{et al.} 2004\(^a\),\(^b\)). BSp66 dimmerizes by disulfide bonds into a higher form named BSp120 (120 kDa) when bovine spermatozoa is cryopreserved (Cesari \textit{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 \textit{in vitro} fertilization.

Materials and Methods

\textbf{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 \textit{in vitro} fertilization were purchased at

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Sperm isolation

Epididymides were obtained from sexually mature (2–3 months old) Mesocricetus auratus (golden hamster). Spermatozoa were dispersed from dissected caput or cauda epididymis 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 10μM 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 (250μl) contains 5×106 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 check 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 (10μl) 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 not 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 electrophorised 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% (w/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 immunoglobulin fluorescein isothiocyanate (FITC)-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 an 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 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 x 10⁶ 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 x g, 15 min) to eliminate unbound antibodies and used to inseminate oocytes with a final concentration of 0.2 x 10⁶ 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 x ). 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 spermatozoa.
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 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).

**Antisera 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 μM), 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.

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

a,bDifferent superscripts are statistically different (P < 0.05).

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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 had 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...
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 antisera 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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