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Low Temperature-Induced Dimerization of the Bovine Sperm Serine Protease, BSp66

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Abstract BSp120 and BSp66 are trypsin-like serine proteases from bovine spermatozoa. The former is active in cryopreserved sperm samples while the latter shows proteolytic activity in recently obtained fresh sperm. Both proteases are immunologically related and co-localize in the apical portion of the sperm head. In Western blots with specific antibodies, sperm samples incubated with reducing agents showed a decrease in the amount of BSp120, while BSp66 was detected with both anti-BSp120 and anti-BSp66 antibodies. BSp120 was evident in frozen intact spermatozoa after 60 days of semen cryopreservation and the kinetic of appearance of this protein was coincident with the decrease in the amount of BSp66. Identical results were obtained by freezing sperm extracts from fresh semen at −20°C. Our results suggest that BSp120 results from disulfide bond-dimerization of BSp66 and that this process may be induced by temperatures below zero in both intact spermatozoa and in sperm extracts. J. Cell. Biochem. 88: 1057–1065, 2003.

Key words: reproduction; bovine spermatozoa; cryopreservation; serine protease

Cryopreservation of gametes is a common practice in reproductive biotechnology. Cellular changes occurring in spermatozoa during storage in liquid nitrogen are currently studied since impaired fertility is observed by comparison with fresh semen [Watson, 2000]. Protein, cholesterol, and lipid composition in plasma membrane suffer modifications during capacitation in porcine spermatozoa. These changes that alter membrane fluidity are mimicked by cryopreservation [Pettit and Burh, 1998] and are explained by membrane protein destabilization induced by freezing [Pérez et al., 1996]. Low temperature-induced alterations in acrosomal structure were also described [Moses et al., 1995; Althouse et al., 1998]. High levels of oxygen reactive species leading to unsaturated fatty acids peroxidation of acrosomal membrane [Bilodeau et al., 2000] and increased calcium influx, initiates a process called "cryocapacitation" and acrosomal reaction prematurely [Esteves et al., 1998; Thundathil et al., 1999; Bailey et al., 2000] by altering sperm proteins mainly on the plasma membrane. Sulfhydryl groups of membrane proteins maintain a dynamic equilibrium with their disulfide counterparts. It has been demonstrated that cooling and freezing-thawing exert marked effects on the sulfhydryl groups of bull sperm membrane proteins [Chatterjee et al., 2001]. Altogether, cold-induced alterations result in a reduction in fertilization rates of cryopreserved mammalian spermatozoa [Moses et al., 1995; Althouse et al., 1998; Pettit and Burh, 1998].

Mammalian fertilization needs that spermatozoa penetrate two oocyte layers which are cumulus oofurus (CO) and zona pellucida (ZP). Two possible mechanisms allow entry through the ZP: mechanical force (sperm motility) and enzymatic hydrolysis [Kohno et al., 1998]. Several serine proteases have been described

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in mammalian spermatozoa including acrosin [Zaneveld et al., 1971; Baba et al., 1994a,b; Kohno et al., 1998]. In our laboratory two antigenically related trypsin-like proteases, BSp120 and BSp66, which are located in the apical portion of bovine sperm head, were recently described [Cesari et al., submitted for publication]. We have preliminary evidence that support a membrane-bound localization of these proteases. Alterations in this bovine sperm proteolytic system were observed when cryopreserved spermatozoa were compared with fresh spermatozoa. Cryopreserved sperm displays BSp120 activity while fresh sperm shows BSp66 activity. However, when fresh sperm extracts were stored at −20°C during more than 30 days, activity of BSp120 become appreciable [Cesari et al., submitted for publication]. In this work we report that BSp66 dimerizes into an active form, BSp120, and that dimerization is induced by low temperatures.

MATERIALS AND METHODS

Materials

Casein, Mr protein standards and other analytical grade chemicals were purchased from Sigma-Aldrich (USA). Azocasein was prepared in our laboratory as described by Kirtley and Koshland [1972]. Polyclonal antibodies against BSp120 and BSp66 were prepared in our laboratory as described [Cesari et al., submitted for publication].

Semen Source

Cryopreserved semen pills were generously provided by the Bovine Artificial Insemination Industry (CIALE SA) and by INTA-Balcarce, Argentina. Each dose (250 μl) contained a final concentration of 50×10^6 spermatozoa/ml. This material was conserved in liquid nitrogen (−196°C). Recently ejaculated (fresh) semen was collected using an artificial vagina, diluted in buffer 10 mM Hepes (pH 7.5) and washed twice by centrifugation at 400 *g* for 10 min at 4°C. Then, spermatozoa were suspended in the same buffer to a final concentration of 50×10^6 spermatozoa/ml.

Cryopreservation of Spermatozoa

One to three ejaculates from 5 bulls housed at the Balcarce Experimental Station of INTA were collected by artificial vagina. Semen was evaluated for percentage of progressively motile spermatozoa and sperm concentration. Only ejaculates containing at least 70% motile spermatozoa and more than 600×10^6 spermatozoa/ml were pooled and diluent was added at 35–37°C to give a concentration of $150–200 \times 10^6$ spermatozoa/ml. The extender used was 230 mM lactose, 5% v/v glycerol, 20% v/v egg yolk, 1 mg/ml streptomycin, and 0.6% mg/ml penicillin. The diluted semen was cooled to 5°C in 1 h. Drops of diluted semen (200 μl) were made on dry ice carbonic holes and after 5 min, transferred to liquid nitrogen at −196°C and stored until used.

Determination of Proteolytic Activity

Routinely, azocasein was used as substrate. The reaction mixture contained 0.5% (w/v) azocasein, 0.1 M Tris-HCl (pH 7.5), and enzyme solution in 300μl final volume. Incubations were performed at 39°C during 4 h and stopped by adding 1 volume of cold 10% trichloroacetic acid (TCA). The assayed tubes were left on ice for 15 min and centrifuged for 15 min at 3000 *g*. Acid-soluble products were detected in the supernatant by measuring absorbance at 335 nm (A_{335}). One unit of activity (U) was defined as the amount of enzyme that produced an increase of 1 in A_{335} under the conditions above described. The conditions to measure trypsin and papain activities were adjusted in order to obtain an increment of $A_{335} = 1$ in 30 min at 39°C. For papain activity reaction mixture contained 1 mM β-mercaptoethanol.

Proteolytic activity on the synthetic oligopeptide Gly-Gly-Arg-α-4-methylcoumaril-7-amide (MCA) was performed in a reaction mixture containing 0.2 mM peptidyl-MCA, 0.1 M Tris-HCl (pH 7.5), and enzyme solution in a final volume of 75 μl. Incubations were performed at 39°C and stopped by adding 2 mM PMSF (phenylmethylsulfonyl fluoride). The degradation product of peptidyl MCA was measured fluorometrically at 460 nm with an excitation filter of 360 nm in a Turner 450 fluorometer. One unit was defined as the variation of fluorescent emission related to the fluorescent signal of a 0.2 mM 7-amido-4-methyl-coumarine solution.

Protein Determination

Protein concentration was determined by the bicinchoninic acid procedure [Smith et al., 1985] using bovine serum albumin as standard.

When samples were obtained after SDS–PAGE, portions of gel corresponding to BSp120 and BSp66 Mr, were sliced and then incubated overnight at 4°C in 100 mM Tris-HCl (pH = 7.5) and homogenized in the same buffer. After centrifugation protein concentration was determined in the supernatant.

Partial Purification of BSp120 and BSp66

Purification of BSp120 and BSp66 was performed as described [Cesari et al., submitted for publication]. BSp120 and BSp66 were obtained from cryopreserved or fresh spermatozoa, respectively.

Polyacrylamide Gel Electrophoresis

Samples were electrophoresed in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS–PAGE) at 4°C and 20 mA/gel, according to Laemmli [1970]. Samples were mixed with 5 × Laemmli sample buffer with or without 1 mM dithiotheritol (DTT) and 25 mM β-mercaptoethanol (β-MCE). Samples were not boiled. Mr were estimated using protein standards: β-galactosidase (116 kDa), bovine serum albumin (66 kDa), and ovoalbumin (43 kDa). Proteins were stained with Coomassie brilliant blue R-250 (CBB).

Disulfide Reduction and S-Carboxymethylation

Samples were incubated in the presence of 10 mM DTT and 0.1% SDS, in 100 mM Tris-HCl (pH 7.5) for 5 min at room temperature. Then, iodoacetamide was added (final concentration 34 mg/ml) and samples were further incubated for 5 min. Excess of DTT, SDS, and iodoacetamide were eliminated by extensive washing with 50 mM Tris-HCl (pH 7.5) and the sample concentrated in Amicon units (10.000). After this treatment the activity was measured using azocasein and Gly-Gly-Arg-MCA and also by zymography. Trypsin and papain activities were assayed as controls.

Zymography

Proteins were fractionated in 7.5% (w/v) polyacrylamide gels containing 0.1% (w/v) gelatin following the method of Hummel et al. [1996]. Electrophoresis was performed as described above. In order to eliminate SDS the gel was washed in 2.5% (v/v) Triton X-100, 5% (w/v) $CaCl_2$ 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 CBB staining.

Western Blotting

Proteins were fractionated in polyacrylamide gels as described and then transferred onto nitrocellulose membranes at 190 mA for 30 min in Trans-Blot SD-cell (Bio-Rad). The transfer solution contained 48 mM Tris/39 mM glycine (pH 9.2) and 20% (v/v) methanol. Membranes were blocked in 25 mM Tris-HCl (pH 7.4), 0.02% (v/v) Tween-20, 5% (w/v) skimmed milk, and 0.02% sodium azide (w/v) (blocking buffer) at room temperature for 2 h. The blocked membranes were incubated with specific antiserum diluted 1:200 in blocking buffer at room temperature for 4 h with gentle shaking and then washed in blocking buffer. The membranes were incubated with alkaline phosphatase-conjugated anti-rabbit IgG diluted 1:7500 in the same buffer for 1 h. After extensive washing, the membranes were immersed in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$), containing 0.33 mg nitrobluetetrazolium and 0.01 mg 5-bromo-4-chloro-3-indolyl phosphate per ml until color development, and stopped with distilled water.

RESULTS

Analysis of BSp120 After Treatment With Reducing Agents

BSp120 was detected and partially purified from cryopreserved bovine spermatozoa while BSp66 was obtained from fresh spermatozoa [Cesari et al., submitted for publication]. During purification of BSp66, the presence of a proteolytic band corresponding to the relative mobility of BSp120 was observed in gelatin zymograms when the extracts were stored at −20°C during 60 days or longer [Cesari et al., submitted for publication]. This result agreed with the evidence obtained in Western blots, indicating that both proteins were present in fresh and cryopreserved spermatozoa, provided that the samples had been stored at temperatures below 0°C [Cesari et al., submitted for publication]. The Mr of BSp120 (120 kDa) is about twice the one of BSp66 (66 kDa). To evaluate whether BSp120 could result from dimerization of BSp66, we treated partially purified BSp120 isolated from cryopreserved intact spermatozoa with the reducing agents β-MCE or DTT. Then, the samples were analyzed

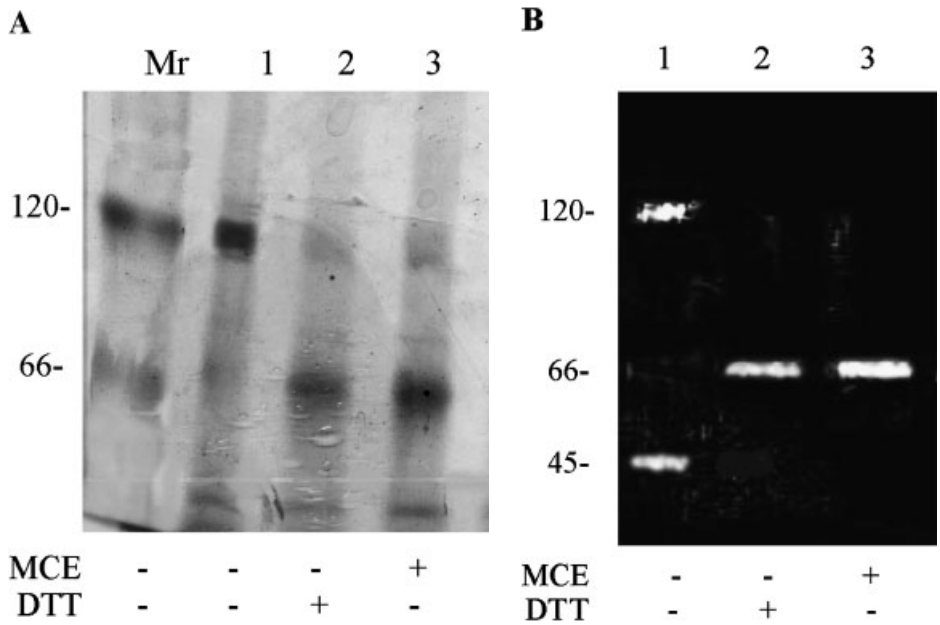

Fig. 1. Effect of reducing agents on BSp120. SDS–PAGE (**A**) and Zymography (**B**) profiles of partially purified BSp120 from cryopreserved spermatozoa obtained after benzamidine-Sepharosa chromatography as described [Cesari et al., submitted for publication]. Mr: molecular mass standards (kDa); **lane 1**, without reducing agent; **lane 2**, 1mM DTT; **lane 3**, 25 mM 2-β-mercaptoethanol.

by SDS–PAGE (Fig. 1A) and zymography (Fig. 1B). After treatment with the reducing agents, the protein band corresponding to BSp120 decreased, while the intensity of the one corresponding to BSp66 increased (Fig. 1A, lanes 1–3). As shown in the zymogram, the activity of BSp120 was no longer detected after the samples were treated with the reducing agents concomitantly with the appearance of BSp66 activity (Fig. 1B, lanes 1–3). The 45 kDa gelatinolytic band, which was inactivated after treatment with the reducing agents (Fig. 1A, lane 1–3), was attributed to acrosin. We had previously demonstrated that acrosin (45 kDa) co-purified with BSp120, as it was recognized by specific anti-acrosin antibodies [Cesari et al., submitted for publication]. The identity of BSp120 and BSp66 was confirmed by Western blotting using specific antibodies (Fig. 2). After incubation with β-MCE or DTT only BSp66 was detected while BSp120 was no longer evident using either anti-BSp66 or anti-BSp120 specific antibodies (Fig. 2A,B). This result suggests that BSp120 may be a dimer of two BSp66 subunits connected by disulfide bridges.

In order to verify if cysteine residues in BSp66 were required not for the activity but for the homodimer formation, the proteins were reduced and S-carboxymethylated, and the proteolytic activity was measured. Figure 3 shows that BSp66 is still active after reduction and carboxymethylation indicating that disulfide bonds are not necessary for protein activity. Identical results were obtained when BSp66 proteolytic activity was assayed in vitro using either azocasein or Gly-Gly-Arg-MCA as substrates (data not shown). Trypsin (negative control) activity was not affected while Papain

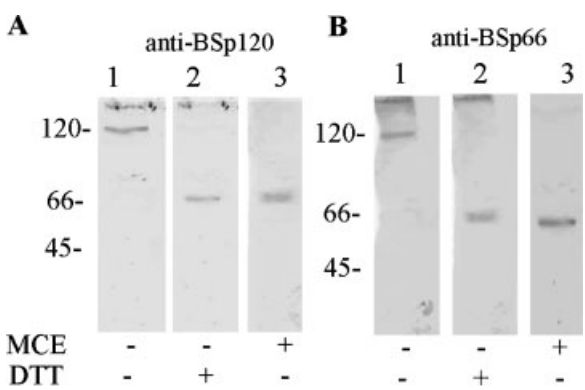

Fig. 2. Effect of reducing agents on the immunological detection of BSp120. Western blot analysis of partially purified BSp120 from cryopreserved spermatozoa (30 μg), incubated with (**A**) anti-BSp120 (1/200) and (**B**) anti-BSp66 (1/200) antibodies. **Lane 1**: Without reducing agent; **lane 2**, 1mM DTT; **lane 3**, 25 mM 2-β-mercaptoethanol. Mr standards are indicated on the left (kDa).

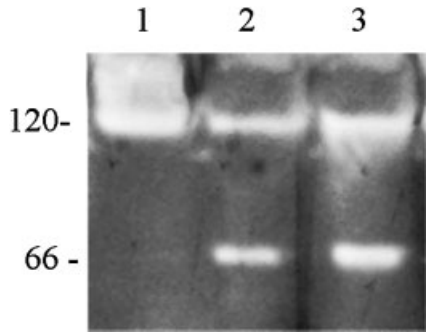

Fig. 3. Activity of the monomer BSp66 after S-carboxymethylation. Zymography of partially purified BSp120 from cryopreserved spermatozoa. Control (**lane 1**), reduction with 10 mM DTT (**lane 2**), and S-carboxymethylation with 34 mg/ml iodoacetamide (**lane 3**) were assayed. Before electrophoresis, samples were extensively washed to remove the excess of reagents. Mr standards are indicated on the left (kDa).

(positive control) activity decreased 98%. Due to high amount of protein loaded in the zymography, BSp120 was not completely reduced to BSp66.

Changes in Protease Structure During Storage at Low Temperatures

To test whether storage of semen samples at −196°C may induce dimerization of BSp66 in intact spermatozoa, we analyzed the presence and activity of BSp120 and BSp66 in fresh sperm at different times after cryopreservation (Fig. 4A). BSp66 was the only band detected in fresh sperm extracts using either anti-BSp120 (Fig. 4C, lane 1) or anti-BSp66 antiserum (not shown). After 60 days of cryopreservation, a protein band corresponding to 120 kDa was also detectable by SDS–PAGE and Western blotting and remained evident at least up to 120 days (Fig. 4A,C, lanes 4 and 5). Accordingly, the

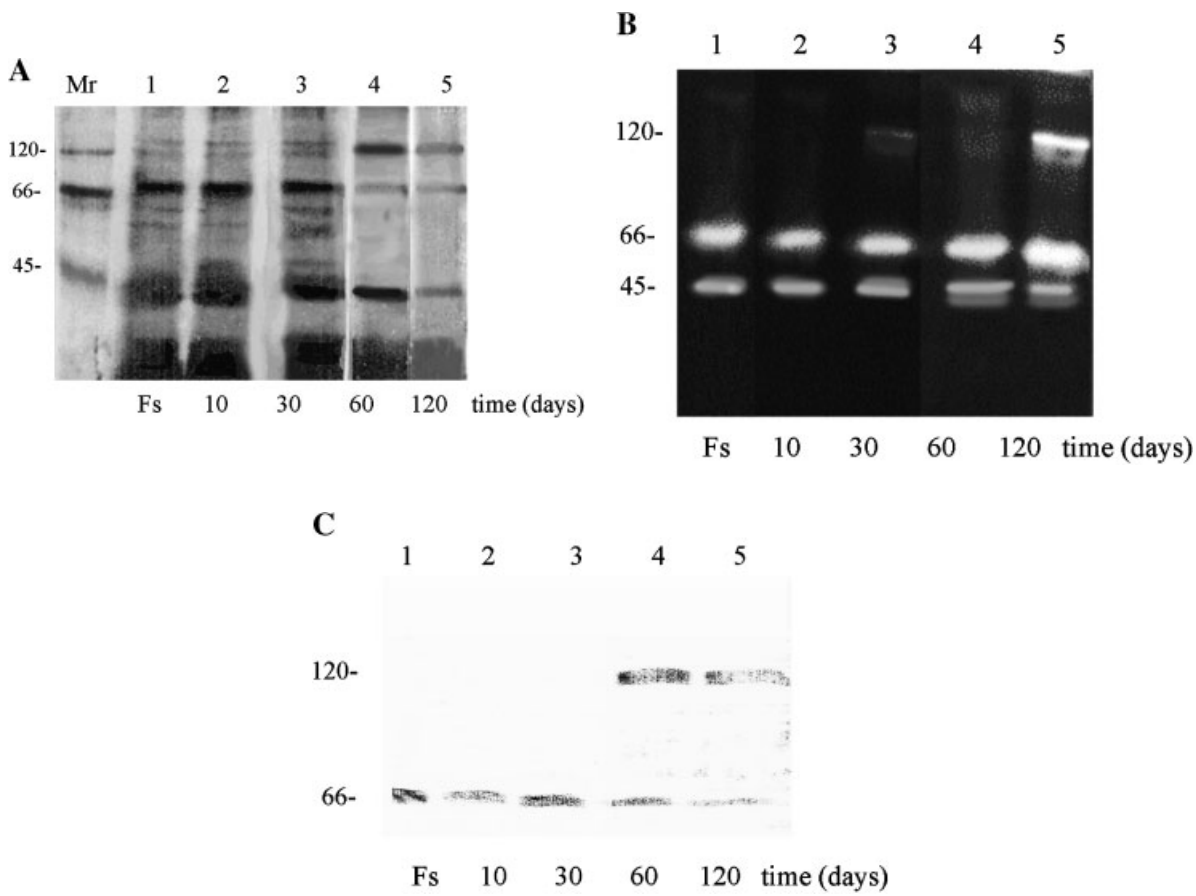

Fig. 4. Effect of cryopreservation (−196°C) on BSp66. Fresh spermatozoa were diluted in extender solution and frozen at −196°C. Then, samples were thawed at different times, spermatozoa extracts (50 μg) were analyzed by SDS–PAGE (**A**), Zymography (**B**), and Western blotting with (**C**) anti-BSp120 antibody (1/200). Electrophoresis was performed under non-reducing conditions. Lane Mr: Molecular mass standards (kDa); **lane 1**, Fresh sperm; **lane 2**, 10 days; **lane 3**, 30 days; **lane 4**, 60 days; **lane 5**, 120 days.

pattern of proteolytic activity showed the appearance of the active band corresponding to BSp120 after 30–60 days of cryopreservation of spermatozoa (Fig. 4B). A remarkable decrease in the amount of BSp66 was also observed after 60–120 days of storage (Fig. 4A,C, lanes 4 and 5). This decrease was not observed in zymograms probably due to the high levels of BSp66 activity in addition to the semiquantitative nature of this method.

In parallel, sperm extracts from fresh spermatozoa were stored at −20°C during different times to investigate the possibility that incubation at low temperatures may induce formation of BSp120 from BSp66 in the absence of intact cells. As observed in cryopreserved spermatozoa, Western blot analysis revealed that the kinetics of appearance of BSp120 was concomitant with the decrease in BSp66 (Fig. 5A). The results observed in gelatin zymograms also evidenced this conversion (Fig. 5B).

These results indicate that dimerization of BSp66 into BSp120 is induced by low temperatures.

Changes in Proteolyitc Activity During Cryopreservation

In order to evaluate whether cryopreservation of intact spermatozoa may result in variation of total proteolytic activity, azocaseinolytic activity was measured in sperm extracts following semen storage at −196°C for different times (Fig. 6). A fivefold increment of total activity was observed after 60 days of cryopreservation, suggesting that freezing/thawing enhances total proteolytic activity in bovine spermatozoa.

On the other hand, when proteolytic activity of partially purified fractions containing BSp120 and BSp66 was measured in the presence of 25 mM β-MCE or 1 mM DTT, conditions that induce the conversion of BSp120 into BSp66, a threefold decrease in activity was observed (not shown). To investigate whether this decrease may be due to lower activity of the monomer (BSp66) as compared to that of the dimer (BSp120), or to a direct or indirect effect of reducing agents on BSp66 active site, both proteases were separated by SDS–PAGE and the fractions of the gel corresponding to BSp120 and BSp66 Mr were sliced from the gel and incubated with azocasein in the presence or absence of β-MCE (Fig. 7). A 55% reduction in the activity of BSp120 was observed after incubation of this protein with β-MCE. Since BSp66 activity was increased by effect of the reducing agent, an inhibitory effect of β-MCE on the intrinsic activity of the monomer, may be discarded. We concluded that the dimeric form is more active than the monomeric one, and as a consequence, at least part of the increment of activity observed during cryopreservation may be attributed to formation of the dimeric form of the protease.

DISCUSSION

BSp120 and BSp66 are proteolytic enzymes of bovine spermatozoa. They share several properties such as trypsin-like proteolytic activity, subcellular localization and cross-reactivity

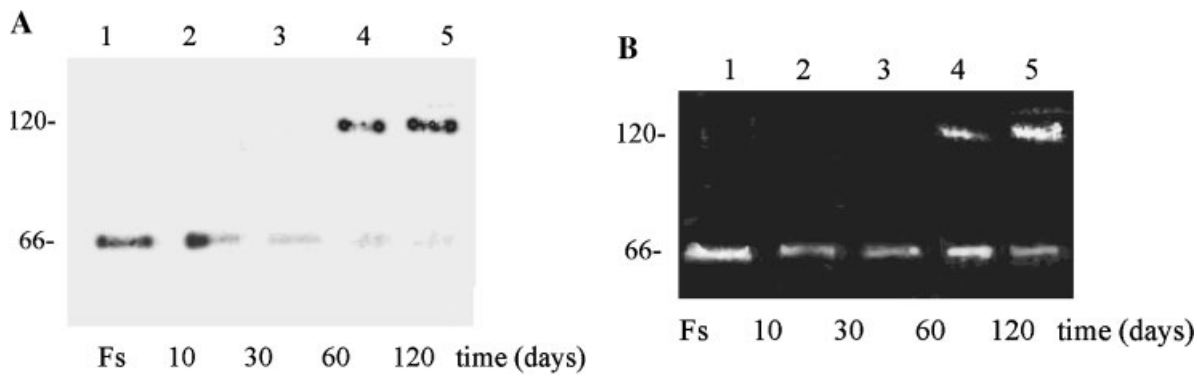

Fig. 5. Effect of below-zero conservation of fresh sperm extracts on BSp66. Protein extracts obtained from fresh spermatozoa were stored at −20°C during different times. Then, 50 μg protein were analyzed by Western blotting with antibodies against (**A**) BSp120 (1/200) and (**B**) Zymography. Electrophoresis was performed under non-reducing conditions. **Lane 1**: Fresh sperm; **lane 2**, 10 days; **lane 3**, 30 days; **lane 4**, 60 days; **lane 5**, 120 days. Mr standards are indicated on the left (kDa).

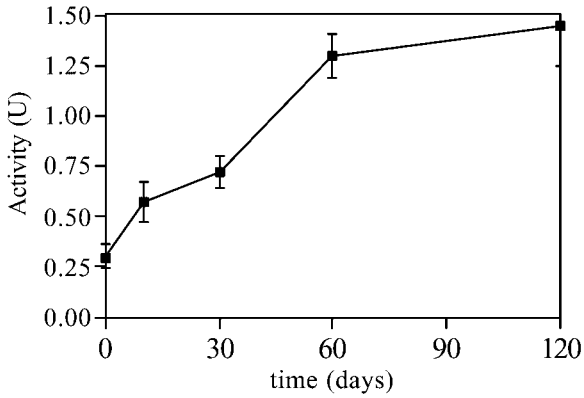

Fig. 6. Changes in total azocaseinolytic activity during cryopreservation of spermatozoa. In parallel to the experiment describe in Figure 3, total activity of sperm extracts (200 μg) was measured using azocasein as substrate. One unit of activity (U) was defined as the amount of enzyme that produced an increase of 1 in A_{335} in 4 h at 39°C. Results are presented as means of three identical assays ± standard deviation (SD).

against specific antibodies [Cesari et al., submitted for publication]. The present study was carried out in order to elucidate the relation existing between BSp120 and BSp66 and the effect of low temperature on this proteolytic system. We have previously observed that BSp120 is mostly active in cryopreserved sperm while BSp66 is active mainly in fresh spermatozoa [Cesari et al., submitted for publication]. In this work we provide evidence about structural modifications of BSp66 during freezing and thawing. We propose that it dimerizes into

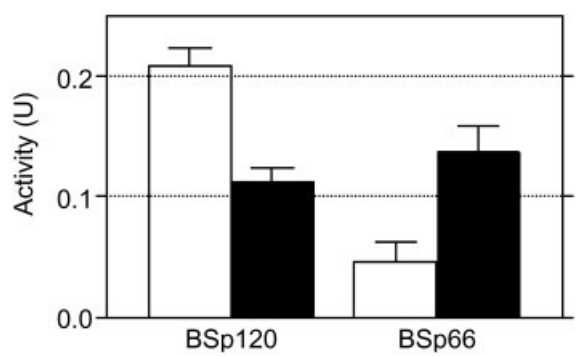

Fig. 7. Effect of β-MCE over BSp120 and BSp66 azocaseinolytic activity. Partially purified BSp120 and BSp66 from fresh spermatozoa stored during 60 days at −20°C were separated by SDS–PAGE. Gel fractions containing each protease were incubated over azocasein in the presence (filled bars) or absence (empty bars) of 25 mM β-MCE, during 16 h at 30°C. Protein concentration of both gel fractions was identical (0.25 mg/ml). Activity data (U defined in Fig. 5) are presented as means (n = 3) ± SD.

BSp120 by means of disulfide-bond formation, as evidenced by treatment of BSp120 with reducing agents (Figs. 1 and 2). BSp120 and BSp66 displayed enzymatic activity before and after incubation with reducing agents, respectively (Fig. 1B). In agreement with these results, S-reduction and carboxymethylation did not affect BSp-66 activity (Fig. 3) indicating that disulfide bridges are not required for its enzymatic activity. When the activity was quantified, a decrease in total proteolytic activity was found in partially purified fractions from cryopreserved spermatozoa by effect of reducing agents (not shown), which was attributed in part, to a reduction of the activity as a consequence of dissociation of BSp120 into BSp66 by effect of β-MCE (Fig. 7). Antibodies obtained in our laboratory against BSp120 and BSp66 confirmed the conversion of BSp120 into BSp66 (Fig. 2). Both antibodies cross-reacted with the same protein species in Western blots in the presence or absence of reducing agents (compare Figs. 2A and 2B), confirming the antigenic relationship between these proteins [Cesari et al., submitted for publication]. Conversion of BSp66 into BSp120 was observed after storage of semen samples at −196°C for 60 days (Fig. 4C) as well as in protein extracts from fresh spermatozoa conserved at −20°C (Fig. 5A). These results explain the occurrence of BSp120 activity in sperm extracts stored below 0°C for a long time [Cesari et al., submitted for publication]. Modifications induced by low temperatures on these proteases were independent from rearrangements occurring in the plasma membrane after freezing and thawing [Pettit and Burh, 1998] as shown by the identical kinetics of conversion of BSp66 into BSp120 displayed in intact cryopreserved spermatozoa or frozen sperm extracts (Figs. 4 and 5).

Decrease in fertility after cryopreservation of spermatozoa was explained by factors affecting the proportion of survivors and factors influencing the functional status of survivors [Watson, 2000]. In the second group, the considerable reduction of intact acrosomes and acrosin activity can be considered [Esteves et al., 1998]. However, the methodology commonly employed for clinical evaluation of acrosin activity is based on determination of sperm amidase activity on the peptide substrate *N*α-benzoyl-DL-arginine p-nitroanilide (BAPNA) [Kennedy et al., 1989; Pleban et al., 1990; Glogowski et al., 1998]. If as proposed, other trypsin-like serine

proteases are present in spermatozoa, total proteolytic activity of intact spermatozoa or total sperm extracts should not be attributed only to acrosin. In this work we obtained higher total protease activity in frozen sperm extracts than in fresh sperm extracts. This increment of activity correlated with protease dimerization kinetics (Fig. 6) suggesting that BSp120 may be more active than BSp66 under these conditions. This hypothesis was consistent with the results obtained after electrophoretic fractionation of BSp120 and BSp66 and determination of protein activity (Fig. 7). Alternatively, another protease(s) may be activated by cryopreservation, and/or endogenous protease inhibitors may be inactivated. The physiological significance of these modifications may be interpreted as related to sulfhydryl modifications linked to freezing and thawing [Chatterjee et al., 2001]. Removal of coating-components on membrane sulfhydryl groups may expose BSp66 sulfhydryl groups allowing dimerization into BSp120. It is also possible that this dimerization is a phenomenon that occurs during natural capacitation, and that premature capacitation of frozen-thawed spermatozoa mimics this event. Experiments aimed to evaluate the dimerization kinetics along the different steps of capacitation are needed to address this issue.

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