BIOLOGY OF REPRODUCTION **76,** 424–432 (2007)
Published online before print 22 November 2006.
DOI 10.1095/biolreprod.106.055624

Induction of Epididymal Boar Sperm Capacitation by pB1 and BSP-A1/-A2 Proteins, Members of the BSP Protein Family[1]

Marie-France Lusignan,[3] Annick Bergeron,[3] Marie-Hélène Crête,[3] Claude Lazure,[4] and Puttaswamy Manjunath[2,3]

Guy-Bernier Research Center,[3] Maisonneuve-Rosemont Hospital and Department of Medecine, University of Montreal, Montreal, Quebec, Canada H1T 2M4
Clinical Research Institute of Montreal,[4] Montreal, Quebec, Canada H2W 2T2

ABSTRACT

A family of proteins designated BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa, collectively called BSP (bovine seminal plasma) proteins, constitute the major protein fraction of bull seminal plasma. BSP proteins can stimulate sperm capacitation by inducing cholesterol and phospholipid efflux from sperm. Boar seminal plasma contains one homologous protein of the BSP family, named pB1; however, its physiological role is still unknown. In the current study, we report a novel method to purify pB1 from boar seminal plasma by chondroitin sulfate B-affinity chromatography and reverse-phase-high performance liquid chromatography. We also studied the effect of pB1, BSP-A1/-A2, and whole boar seminal plasma on boar sperm capacitation. Boar epididymal sperm were washed, preincubated in noncapacitating medium containing pB1 (0, 2.5, 5, 10 or 20 μg/ml), BSP-A1/-A2 (0 or 20 μg/ml) proteins, or whole seminal plasma (0, 250, 500, or 1000 μg/ml), then washed and incubated in capacitating medium. Acrosomal integrity was assessed by chlortetracycline staining. The status of sperm capacitation was evaluated by the capacity of sperm to undergo the acrosome reaction initiated by the addition of the calcium ionophore, A23187. The pB1 and BSP-A1/-A2 proteins increased epididymal sperm capacitation as compared with control (sperm preincubated without proteins). This effect reached a maximum level at 10 μg/ml pB1 and at 20 μg/ml BSP-A1/-A2 (2.3- and 2.2-fold higher than control, respectively). Whole boar seminal plasma did not induce sperm capacitation. In addition, pB1 bound to boar epididymal sperm and was lost during capacitation. These results indicate that BSP proteins and their homologs in other species induce sperm capacitation in a similar way.

acrosome reaction, fertilization, male reproductive tract, sperm, sperm capacitation

INTRODUCTION

Bovine seminal vesicles secrete a family of lipid-binding proteins designated BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa (collectively called BSP [bovine seminal plasma] proteins; accession numbers: P02784, P04557, P81019) [1, 2]. Since BSP-A1 and BSP-A2 have an identical amino acid sequence, with their difference residing only in their degree of glycosylation, they are considered a single chemical entity, called BSP-A1/-A2, also called PDC-109 [3]. BSP proteins contain two similar fibronectin type II domains arranged in tandem [3–5], which mediate binding to choline phospholipids [6], high density lipoproteins (HDL; [2, 7]), glycosaminoglycans (GAGs; such as heparin, heparan sulphate, and chondroitin sulfate B [8, 9]), and gelatin [10]. These binding properties can be used to isolate BSP proteins from bovine seminal plasma [8–10].

BSP proteins play a role in bovine sperm capacitation (reviewed in [11]), which is a multistep process that sperm must undergo to be able to fertilize eggs. This process consists of many biochemical and physiological changes that occur in sperm during their transit through the female reproductive tract [12–14]. The general mechanism of capacitation proposed by our laboratory includes modification of the sperm membrane by BSP proteins [11]. At ejaculation, BSP proteins are mixed with sperm, bind to choline phospholipids of the sperm membrane, and stimulate phospholipid and cholesterol efflux from the sperm membrane [15]. Cholesterol removal from the sperm membrane is important for the capacitation process (reviewed in [16]). As sperm reach the oviduct, they encounter follicular and oviductal fluids. Heparin-like GAGs and HDL are components of the oviductal and follicular fluids, found in the female reproductive tract, which induce sperm capacitation [17–25]. Interestingly, BSP proteins potentiate capacitation induced by heparin [26], GAGs [9], and HDL [25]. HDL can then interact with sperm-bound BSP proteins, which would result in further removal of phospholipids and cholesterol, leading to the capacitation state [27]. Alternatively, heparin-like GAGs could interact with sperm-bound BSP proteins and promote capacitation [9], which involves protein tyrosine phosphorylation [28]. In addition, BSP proteins also play a role in the formation of the oviductal sperm reservoir because they enable sperm to bind the oviductal epithelium [29, 30].

Proteins homologous to BSP proteins have been found in the seminal plasma of other mammalian species such as stallion [31, 32], goat [33], bison [34], ram [35], and boar [36]. BSP-like antigens have also been found in mouse, human, hamster, and rat semen or seminal vesicle secretions [2, 37]. Recently, three new BSP related genes (*BSPH4*, *BSPH5*, and *BSPH6*) have been discovered in the bovine [38]. In addition, an exhaustive genomic search indicated the presence of BSP-related DNA sequences in human, rat, mouse, chimpanzee, dog, horse, and rabbit [38]. However, the role of BSP homologs in other mammalian species is not documented.

One BSP protein homolog, pB1 (accession number: NP 998997), has been isolated from boar seminal plasma to date [32, 36]. Protein pB1 represents ~1% of total boar seminal plasma proteins [39], contains 105 amino acids, possesses different glycoforms, and has a molecular weight of 12.6 kDa,

[1]Supported by the Canadian Institutes of Health Research.
[2]Correspondence: Puttaswamy Manjunath, Centre de Recherche Guy-Bernier, Hôpital Maisonneuve-Rosemont, 5415 boulevard de l'Assomption, Montreal, QC, Canada H1T 2M4. FAX: 1 514 252 3430; e-mail: puttaswamy.manjunath@umontreal.ca

Received: 14 July 2007.
First decision: 3 September 2006.
Accepted: 16 November 2006.

ISSN: 0006-3363. http://www.biolreprod.org

or 13.0–13.4 kDa for the glycosylated forms [36, 40]. The pB1 and BSP proteins share 36%–65% sequence similarity. Similar to BSP proteins, pB1 contains a tandem of fibronectin type II domains and binds to heparin, chondroitin sulfate, phosphorylcholine [36, 41], and epididymal boar sperm [42]. However, the physiological role of pB1 is unknown.

The mechanism of boar sperm capacitation is not fully elucidated. As in many mammals, boar sperm capacitation requires calcium [43] and bicarbonate (HCO_3^-) [44], which induce a reorganization of the membrane lipid architecture [45, 46] and an increase in sperm motility [47]. Boar sperm capacitation correlates with cholesterol efflux from the sperm plasma membrane [48] and with tyrosine phosphorylation [49–51]. The presence of oviductal fluid in the capacitating medium increases the proportion of capacitated and acrosome-reacted boar sperm [52]. The nonsulfated GAG, hyaluronan, can also induce boar sperm capacitation, but without inducing acrosome reaction [53], while heparin alone cannot induce boar sperm capacitation in vitro [52]. Further studies are required in order to understand the molecular mechanisms involved in boar sperm capacitation.

In the present study, we hypothesized that proteins of the BSP family can stimulate boar sperm capacitation. First, we isolated and purified pB1 from whole porcine seminal plasma by a new, efficient method (chondroitin sulfate B-affinity chromatography followed by reverse-phase [rp]-high performance liquid chromatography [HPLC]), and tested whether pB1 could potentiate the capacitation of epididymal porcine sperm. Second, we evaluated the effect of whole boar seminal plasma on sperm capacitation. Third, we tested the hypothesis that BSP-A1/-A2 isolated from bovine seminal plasma could also potentiate the capacitation of epididymal boar sperm. Fourth, we evaluated the fate of pB1 during capacitation.

MATERIALS AND METHODS

Materials

Chondroitin sulfate B, BSA, calcium ionophore A23187, and Freund adjuvant (complete and incomplete) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Acrylamide and bisacrylamide were purchased from ICN (Mississauga, ON, Canada). Affi-Gel 15, SDS, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, and other electrophoresis products were from Bio-Rad (Mississauga, ON, Canada). Immobilon-P polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Nepean, ON, Canada). Low Molecular Weight Calibration kit was from GE Healthcare (Baie d'Urfé, QC, Canada). Western Lightning Chemiluminescence Reagent kit was from Perkin-Elmer Life Sciences (Boston, MA). HPLC-grade trifluoroacetic acid (TFA) was from J.T. Baker (Phillipsburg, NJ). All other chemicals used were of ultra-pure grade and were obtained from local suppliers.

Boar testes and epididymides were obtained from Abattoir Hébert (Ste-Hélène-de-Bagot, QC, Canada). Whole boar seminal plasma was obtained by centrifuging a pool of 3–4 complete ejaculates, obtained from FMénard Inc. (Ange-Gardien, QC, Canada), at 1500 × *g* for 15 min. The supernatant was further centrifuged at 14 000 × *g* for 15 min to obtain clear seminal plasma. The seminal plasma was preserved at −80°C until used. Boar seminal plasma total proteins were prepared by alcohol precipitation [35].

Chondroitin Sulfate B-Agarose Affinity Chromatography

Chondroitin sulfate B was previously coupled to Affi-Gel 15, as previously described [9], with the exception that about 28.5 mg of commercial chondroitin sulfate B was coupled to 7.5 ml of Affi-Gel 15. All purification steps were performed at 4°C. Frozen seminal plasma (3 ml) was thawed, centrifuged at 10 000 × *g* for 10 min, and the supernatant was loaded onto a chondroitin sulfate B column (1.0 × 8.6 cm), previously equilibrated with 10 volumes of 50 mM PBS. Once the sample entered the column, the flow was stopped for 20 min to allow proteins to bind. Following this step, the column was washed with PBS to eliminate unadsorbed and retarded proteins. The bound proteins were then successively eluted with PBS containing 1 M NaCl, and with PBS containing 1 M NaCl and 7 M urea. Fractions of 2 ml were collected at a flow rate of 30 ml/h, and their absorbance at 280 nm was measured. Fractions under each protein peak were pooled, dialyzed against PBS, and frozen at −20°C, or dialyzed against 50 mM ammonium bicarbonate and lyophilized.

Reverse-Phase-HPLC

The proteins were analysed by rp-HPLC, as described previously [35]. Lyophilized proteins from fraction 3 (100 μg) of the chondroitin sulfate B-agarose chromatography step were dissolved in 0.1% TFA containing 6 M guanidine-HCl. The protein solution was centrifuged at 10 000 × *g* for 10 min, and the supernatant was loaded onto the column. The proteins were eluted using a gradient of 0.1% TFA in acetonitrile. Fractions of 1.0 ml were collected at a flow rate of 1 ml/min. The eluted proteins were monitored at 235 nm and dried under vacuum for biochemical analysis and capacitation studies.

Culture Medium

The capacitation medium (CM) was a modified Tyrode medium [50], and was composed of 96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 10 μg/ml phenol red, 20 mM Hepes, 5 mM D-glucose (+), 21.6 mM sodium lactate, 1 mM sodium pyruvate, and 15 mM $NaHCO_3$. Sodium lactate and sodium pyruvate were added before use, and the medium was equilibrated at 39°C at least 1 h before the experiments in a humidified atmosphere with 5% CO_2. The noncapacitating medium (NCM) had the same composition as the CM, except that it contained 120 mM NaCl and no $CaCl_2$ or $NaHCO_3$ [54]. NCM was incubated in the same incubator in air-tight sealed flasks to prevent the formation of bicarbonate. The gas phase in the air-tight-sealed flasks was refreshed outside the incubator every 15 min [50]. The pH of the media was adjusted to 7.4 with NaOH.

Sperm Capacitation

Approximately 1 h after the testes were collected, epididymal sperm were recovered in a plastic tube by making small incisions in the cauda of the epididymides. Sperm were then washed twice (350 × *g* for 10 min) in 10 volumes of NCM and resuspended in NCM in order to adjust the sperm concentration to 80×10^6 sperm/ml. A total of 750 μl of this dilution was preincubated with 750 μl of NCM containing different concentrations of pB1 protein (final concentrations: 0, 2.5, 5, 10, and 20 μg/ml), BSP-A1/-A2 protein (final concentrations: 0 and 20 μg/ml), or different volumes of whole boar seminal plasma (corresponding to final protein concentrations of 0, 250, 500 and 1000 μg/ml, as determined by the Lowry method) for 20 min in air-tight-sealed tubes. Sperm were then washed twice (350 × *g* for 10 min) in 10 volumes of NCM, resuspended in CM or NCM at a sperm concentration of 40×10^6 sperm/ml, and incubated at 39°C for 4 h in a humidified atmosphere containing 5% CO_2.

Evaluation of Sperm Capacitation

After 0 and 4 h incubation, 1 μl of a 0.2 mM ionophore A23187 solution in dimethyl sulfoxide (DMSO) or DMSO alone (as control) was added to 100 μl of sperm suspension and incubated for 30 min. Acrosome reaction in capacitated sperm was induced by ionophore A23187 [51]. After incubation with ionophore, 1 μl of a 2 mg/ml protamine solution was added to samples, which were incubated for an additional minute to prevent sperm head-to-head agglutination caused by in vitro capacitation [55, 56].

Acrosome integrity was evaluated with the chlortetracycline (CTC) assay, as described for boar by Wang et al. [57]. Briefly, cysteine (5 mM) and CTC (750 μM) were dissolved in Tris-NaCl buffer (20 mM Tris base and 130 mM NaCl), pH 7.8, and the solution was kept at 4°C in the dark until use. An aliquot of 15 μl of sperm suspension, 22.5 μl of CTC solution, and 0.75 μl of glutaraldehyde (12.5%) were mixed in a well preheated at 39°C. Two 15 μl droplets of this mixture were placed on a slide and covered with a cover slip. The slides were kept in the dark at 4°C in a humid environment. The slides were then observed with a Leitz Diaplan fluorescent microscope (Leitz) under blue-violet illumination (excitation at 330–380 nm, emission at 420 nm; magnification 100×/1.25 oil immersion objective), and 100 sperm were counted on each droplet for a total of 200 sperm per slide. Two samples per treatment were assayed. Sperm were classified into three categories according to their fluorescence pattern: acrosome-reacted sperm, with only a small fluorescent line at the equatorial segment; non-acrosome-reacted sperm (noncapacitated sperm, pattern F), with fluorescence everywhere on the head; and capacitated but acrosome-intact sperm (bright anterior head with a faintly fluorescent postacrosomal region, pattern B) [57]. Because pattern B is not a true reflection of capacitation [51], we determined the percent capacitated

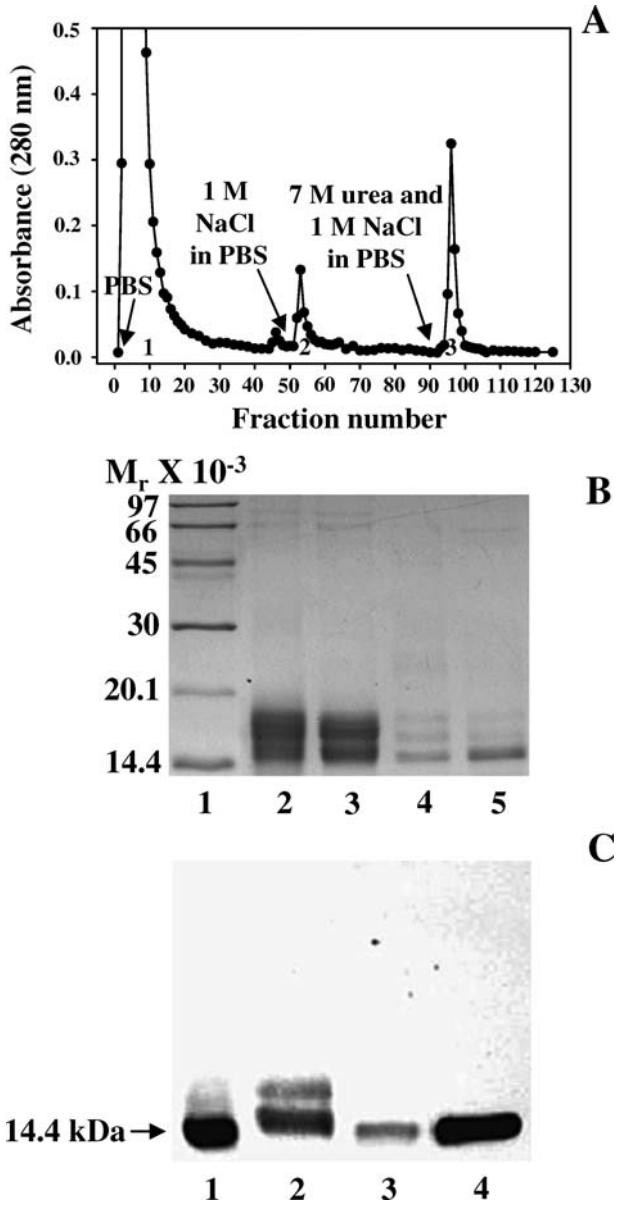

FIG. 1. Isolation and characterization of pB1 protein from boar seminal plasma. **A**) Elution profile of boar seminal plasma proteins on chondroitin sulfate B-agarose. **B**) SDS-PAGE pattern of chondroitin sulfate B-agarose-eluted fractions. Protein samples (15 μg) were reduced, denatured, separated on 15% polyacrylamide gel, and stained with Coomassie blue R-250. Lane 1: Low molecular weight standard; lane 2: total boar seminal plasma proteins; lanes 3–5: proteins from fractions 1, 2 and 3, respectively. **C**) Immunoblot analysis of chondroitin sulfate B-agarose-eluted fractions with anti-pB1 antiserum. Protein samples were separated by SDS-PAGE, transferred to immobilon-P, and probed with antiserum directed against pB1. Lane 1: Total boar seminal plasma proteins (3 μg); lanes 2–4: proteins from fraction 1 (3 μg), fraction 2 (1 μg), and fraction 3 (0.5 μg), respectively.

sperm by subtracting the percent of spontaneous acrosome-reacted sperm (without ionophore) from the percent of acrosome-reacted sperm induced by the ionophore. It is well established that only capacitated sperm undergo the acrosome reaction following induction with ionophore [51].

Binding Studies of pB1 to Sperm Membrane Proteins

After 0 and 4 h incubation in CM, 500 μl of sperm suspension preincubated with 0 or 10 μg/ml pB1 was centrifuged at 12 000 × *g* for 5 min to remove sperm from CM. The sperm pellets were washed 3 times in PBS at 12 000 × *g* for 8 min. Sperm membrane protein extracts were prepared as described previously [58], and were stored at −20°C until use.

Viability

Prior to staining and drying, randomly selected slides were examined subjectively using light microscopy to verify sperm motility. Viability was established with eosin/nigrosin coloration, as described by Dott and Foster [59]. Two samples per treatment and 200 sperm per sample were assayed.

Generation of an Antiserum Against pB1

A peptide of 15 amino acids corresponding to the C-terminal amino acids of pB1 (TVTPNYDRDRAWKYC) was synthesized and conjugated to keyhole limpet hemocyanin at the Sheldon Biotechnology Center (Montreal, QC, Canada). New Zealand rabbits were injected hypodermically with a mixture of 200 μg conjugated peptide dissolved in 100 μl 50 mM PBS, 400 μl sterile 0.9% NaCl, and 500 μl Freund complete adjuvant. Boosts were performed at 10-day intervals over a period of 88 days, with 200 μg of the same antigen in the same mixture, with the exception that Freund complete adjuvant was replaced by Freund incomplete adjuvant. Bleedings were performed 15 days after each injection. Antiserum from the third boost was used for the present study. Animals were treated in accordance with the guidelines of the Canadian Council of Animal Care.

SDS-PAGE and Immunoblotting

Proteins were precipitated with trichloroacetic acid (15% final concentration), reduced, denatured, and separated in 15% polyacrylamide gels, according to Laemmli [60], using the Mini Protean III apparatus from Bio-Rad. Proteins in the gel were then either stained with Coomassie blue or transferred electrophoretically to PVDF membranes, as described previously [61], using a Trans-Blot apparatus from Bio-Rad for immunodetection. Proteins were probed with antiserum directed against pB1 (1:1000). Molecular mass was estimated by comparison with the Low Molecular Weight Calibration kit containing phosphorylase b (M_r = 97 000), albumin (M_r = 66 000), ovalbumin (M_r = 45 000), carbonic anhydrase (M_r = 30 000), trypsin inhibitor (M_r = 20 100), and αlactalbumine (M_r = 14 400).

Protein Sequencing

Following the transfer of proteins that were separated by SDS-PAGE to an Immobilon-P membrane, the Coomassie blue-stained protein band was cut and placed in the sequenator reactor. Sequencing was carried out, as described previously [62], using an Applied Biosystems Procise sequencer (model 494; Applied Biosystems).

Protein Assay

The protein content of the samples was measured by a modified Lowry method [63] or by weighing using a Cahn microbalance (Model C-31; Fisher Scientific, St.-Laurent, QC, Canada).

Data Analysis

The data for sperm function analysis were transformed as a percentage of motility, viability, or acrosomal integrity before analysis. Data were tested for normal distribution with the Shapiro-Wilk test. All data were normally distributed, except where indicated. Data that were not normally distributed were transformed using the arcsin square root in order to obtain a normal distribution before analysis. The data were tested for significant differences by ANOVA. Significant differences among treatments were determined with the protected Fisher least significant difference test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Chondroitin Sulfate B-Agarose Chromatography of Whole Boar Seminal Plasma

Figure 1A shows the chondroitin sulfate B-agarose chromatography pattern of boar seminal plasma proteins. The proteins were eluted in three peaks: unadsorbed (fraction 1); eluted with 1 M NaCl (fraction 2); and urea eluted (fraction 3).

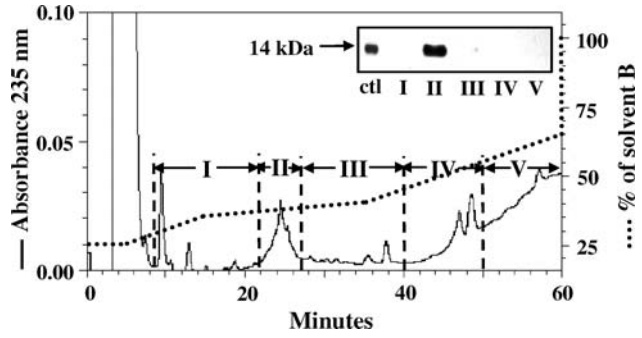

FIG. 2. Reverse-phase HPLC pattern of chondroitin sulfate B-agarose chromatography fraction 3. Protein was prepared as described in *Materials and Methods* and eluted using a 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) buffer system. Elution was isocratic at 25% solvent B for 5 min, followed by a first gradient of 25%–35% solvent B in 10 min, a second gradient of 35%–40% solvent B in 20 min, and a third gradient of 40%–65% solvent B in 25 min. The inset represents the immunoblot pattern of a 1:10 dilution of fractions I and III–V, or a 1:25 dilution of fraction II using an antiserum directed against pB1. Lanes I–V: fractions I-V, respectively. ctl, Total boar seminal plasma proteins (3 μg).

The recovery of the total proteins was about 85% of the starting material following chondroitin sulfate B-agarose chromatography. SDS-PAGE analysis of fraction 1 showed that the majority of proteins did not bind to the column (Fig. 1B). A group of proteins with molecular masses from 14 to 18 kDa were found in fractions 1, 2, and 3 (Fig. 1B, lanes 3, 4, and 5, respectively). A protein with an apparent molecular mass of 14 kDa was predominant in fraction 3 (Fig. 1B, lane 5). Immunoblot experiments showed that the pB1 protein was found in all the fractions eluted from the chondroitin sulfate B-agarose chromatography (Fig. 1C). In fraction 1 (Fig. 1C, lane 2), there were two protein bands that were detected by anti-pB1 antiserum, although the majority of pB1 was found in fraction 3 (Fig. 1C, lane 4). Since fraction 1 still contained pB1, as shown by immunoblot, and because our goal was to accumulate a large quantity of pB1, fraction 1 was rechromatographed on the chondroitin sulfate B-agarose column

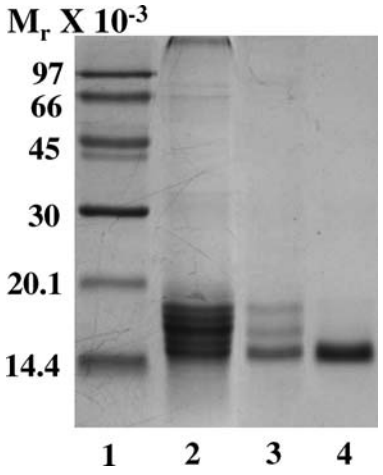

FIG. 3. SDS-PAGE characterization of rp-HPLC fraction II. Protein samples were reduced, denatured, separated on 15% polyacrylamide gel, and stained with Coomassie blue R-250. Lane 1: low molecular weight standard; lane 2: total boar seminal plasma proteins (15 μg); lane 3: elution fraction 3 from chondroitine sulfate B affinity chromatography (15 μg); lane 4: rp-HPLC fraction II (5 μg).

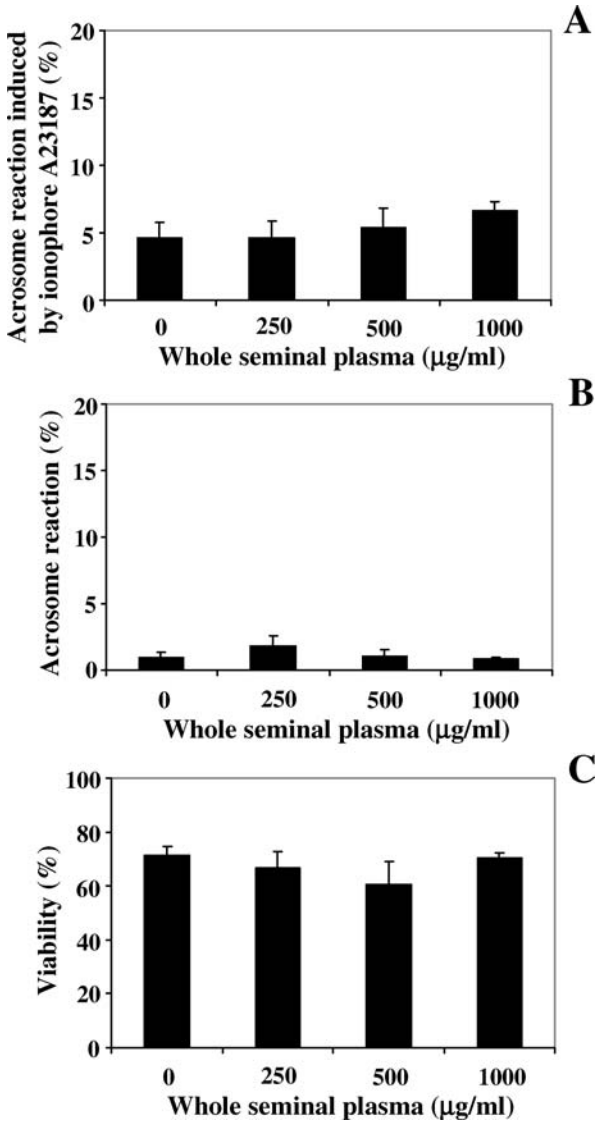

FIG. 4. Effect of whole boar seminal plasma on epididymal sperm capacitation, acrosome reaction, and viability. Cauda epididymal sperm were preincubated for 20 min with or without whole seminal plasma and incubated in CM for 4 h. **A**) Percent of acrosome-reacted sperm induced by ionophore A23187. **B**) Percent of acrosome-reacted sperm without ionophore A23187. **C**) Percent viability. Results represent mean ± SD of 4 experiments that included 2 samples per experiment and 200 sperm assayed per sample.

(data not shown) in order to recover more pB1. The recovery of pB1 was of about 30%–35% for the first run and 20%–35% for the second run.

Purification of pB1 by rp-HPLC

The pB1 protein present in fraction 3 was further purified by rp-HPLC (Fig. 2). The first broad peak that eluted around 5 min contained only salts (data not shown). The elution pattern of fraction 3 showed the presence of a major protein peak (II) that eluted around 25 min. Protein fractions were pooled as described in Figure 2 and submitted to immunoblot analysis with antiserum directed against pB1 (Fig. 2, inset). As shown by immunoblot (Fig. 2, inset, lane II), protein peak II corresponds to pB1. The SDS-PAGE pattern of rp-HPLC fraction II shows that it corresponds to 1 protein band of 14

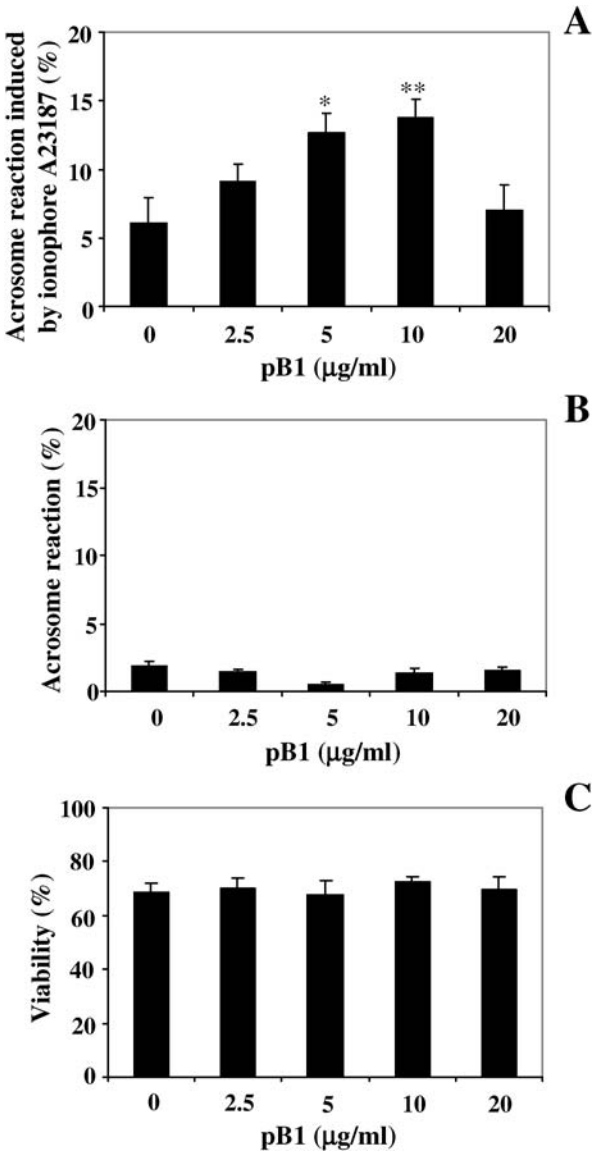

FIG. 5. Effect of pB1 on epididymal sperm capacitation, acrosome reaction, and viability. Cauda epididymal sperm were preincubated for 20 min with or without pB1 protein and incubated in CM for 4 h. **A**) Percent of acrosome-reacted sperm induced by ionophore A23187. **B**) Percent of acrosome-reacted sperm without ionophore A23187. **C**) Percent viability. Results represent mean ± SD of 4 experiments that included 2 samples per experiment and 200 sperm assayed per sample. Significant differences vs. control (without protein): $*P < 0.05$; $**P < 0.01$.

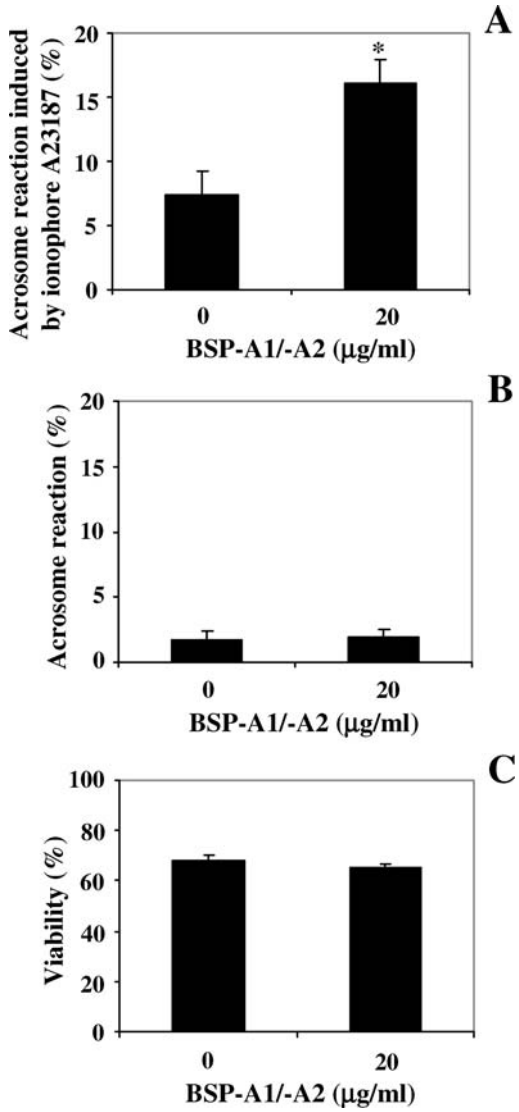

FIG. 6. Effect of BSP-A1/-A2 protein on capacitation, acrosome reaction, and viability of boar epididymal sperm. Cauda epididymal sperm were preincubated for 20 min with or without highly purified BSP-A1/-A2 proteins and incubated in CM for 4 h. **A**) Percent of acrosome-reacted sperm induced by ionophore A23187. **B**) Percent of acrosome-reacted sperm without ionophore A23187. **C**) Percent viability. Results represent mean ± SD of 5 experiments that included 2 samples per experiment and 200 sperm assayed per sample. Data were transformed using the arcsin square root in order to obtain a normal distribution before analysis. Significant differences vs. control (without protein): $*P < 0.01$.

kDa (Fig. 3, lane 4). The electrophoresis pattern obtained with whole boar seminal plasma and elution fraction 3 from chondroitin sulfate B-affinity chromatography are shown in Figure 3 (lanes 2 and 3, respectively) for comparison. The protein impurities of 16 and 18 kDa visible in Figure 3 (lane 3) are not present in the rp-HPLC fraction II (Fig. 3, lane 4). Furthermore, the first 15 amino acids at the N-terminal end were identified (DQHLPGRFLTPAITS) and correspond to the N-terminal sequence of pB1 [36].

Capacitation of Epididymal Sperm by Whole Seminal Plasma

Preincubation of epididymal boar sperm with different volumes of whole boar seminal plasma corresponding to 0, 250, 500, and 1000 μg/ml proteins (as determined by the Lowry method) did not increase the acrosome reaction induced by ionophore A23187 as compared to the control preincubated without seminal plasma (Fig. 4A). The spontaneous acrosome reaction (without ionophore; Fig. 4B), as well as the viability of sperm (Fig. 4C), were not affected by the treatments. Sperm motility was near 50% before and during capacitation experiment.

Capacitation of Epididymal Sperm by pB1

Epididymal boar sperm were preincubated for 20 min with the highly purified pB1 protein, washed, reincubated in CM for 4 h, and the acrosome reaction was induced in capacitated sperm by the addition of ionophore A23187. As shown in

Figure 5A, pB1 preincubation allowed the epididymal boar sperm to undergo the acrosome reaction induced by ionophore A23187. This effect was dose-dependent, with a maximum level at 10 μg/ml pB1, which was approximately 2.3 times (13.8%) the stimulation rate of the control (without pB1; 6.0%). However, at higher pB1 concentrations (20 μg/ml), the acrosome reaction induced by ionophore A23187 was equivalent to the level of the control. Spontaneous acrosome reaction (without ionophore) after 4 h incubation with pB1 was equivalent to that of the control (without proteins; Fig. 5B). None of the treatments affected the viability (Fig. 5C) of epididymal sperm. The motility of the sperm at the beginning was around 50%–60%, and did not change significantly throughout capacitation experiments. In addition, 10 μg/ml pB1 did not increase the acrosome reaction induced by ionophore A23187 as compared to the control (without protein) when the sperm were incubated for 4 h in NCM (results not shown).

Capacitation of Epididymal Sperm by BSP-A1/-A2 Protein

We investigated whether or not bovine proteins homologous to pB1 could induce capacitation in boar epididymal sperm. We chose to perform capacitation experiments with BSP-A1/-A2 because it possesses the highest sequence similarity (65%) to pB1 (data not shown). Figure 6A shows that the preincubation of sperm for 20 min with 20 μg/ml of highly purified BSP-A1/-A2 (minimal concentration that allows maximal bovine epididymal sperm capacitation [26]) increased the percent of acrosome-reacted sperm (16.1%) induced by ionophore A23187, which was 2.2-fold higher than that of the control (without protein; 7.4%). As shown in Figure 6B, a 20-min preincubation of epididymal sperm with BSP-A1/-A2 did not stimulate spontaneous acrosome reaction. Sperm motility was around 45%–55% before and during the capacitation experiments. Viability of epididymal sperm was not affected by the treatments (Fig. 6C).

Fate of pB1 after Capacitation

We evaluated the fate of pB1 following capacitation. As shown in Figure 7 (lane 1), a band at 14.4 kDa corresponding to pB1 was present in the sperm extract when sperm were preincubated with pB1. After 4 h in CM, pB1 was still present, but the intensity of the band corresponding to pB1 was decreased (Fig. 7, lane 2). There was no pB1 in protein extracts of sperm preincubated without protein at 0 and 4 h in the CM (Fig. 7, lanes 3 and 4, respectively). Other bands with molecular weights from 28 to 64 kDa are nonspecific bands.

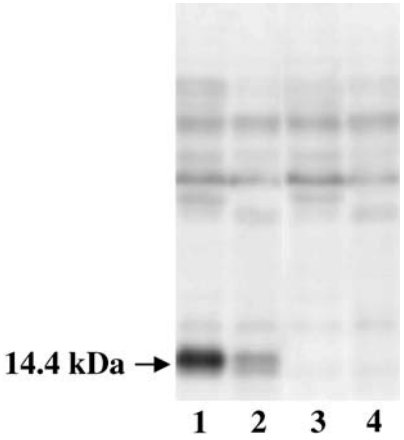

FIG. 7. The fate of pB1 after capacitation. Protein samples were separated by SDS-PAGE, transferred to immobilon-P, and probed with an antiserum directed against pB1. Lanes 1 and 2: protein extracts from sperm preincubated with 10 μg/ml pB1 before and after capacitation, respectively; lanes 3 and 4: protein extracts from sperm preincubated without pB1 before and after capacitation.

DISCUSSION

In this study, we isolated and purified the BSP-homologous protein pB1 from boar seminal plasma with a novel method. The protein concentration in boar seminal plasma is approximately 30 mg/ml [64], and pB1 represents approximately 1% of total boar seminal plasma proteins [39]. The protein recovery was about 50%–70% of total pB1 in boar seminal plasma (150–210 μg of pB1/ml of seminal plasma).

We have previously purified BSP proteins and their homologs from various species using a gelatine-agarose-affinity matrix [10, 33–35]. In preliminary experiments, we observed that, although a portion of pB1 bound to gelatine-agarose, most of it did not interact with the affinity matrix, as indicated by an immunoblot analysis (data not shown). Moreover, several other proteins of boar seminal plasma with molecular weights between 12 and 16 kDa also bound to the column. We chose to isolate pB1 from boar seminal plasma using a chondroitin sulfate B-agarose column because BSP proteins and their homologs have strong affinity for GAGs [9, 41]. Similar to BSP proteins, pB1 also bound strongly to chondroitin sulfate B, and a high concentration of urea was required for the protein to be eluted from the column (Fig. 1A). Protein pB1 eluted mostly in fraction 3, as shown by immunoblot (Fig. 1C, lane 4). The protein was also found in chondroitin sulfate B fractions 1 and 2, indicating that the binding sites in the column for pB1 were probably saturated with the pB1 protein. Two protein bands were observed in chondroitin sulfate B unadsorbed fraction 1 when probed with antibodies against pB1 (Fig. 1C, lane 2). Since pB1 possesses different glycosylated forms [40], it is possible that the higher band corresponds to another glycosylated form of pB1. These different glycosylated forms were not found in chondroitin sulfate B fraction 3, probably because they had less binding affinity for the column due to the glycosylation. Purification of pB1 was tricky because the protein was not soluble in buffers commonly used for protein isolation. The volume of chondroitin sulfate B fraction 3 was 10–15 ml, which was first dialyzed against PBS and concentrated with an ultrafiltration cell in order to inject it into the HPLC column. However, proteins aggregated, and the aggregates were sparingly soluble even in 6 M guanidine-HCl or 7 M urea. The factors that cause protein aggregation in denaturing buffers are not known. Thus, as an alternative, the chondroitin sulfate B fraction 3 was dialyzed against 50 mM ammonium bicarbonate and lyophilized. Lyophilized proteins were dissolved in 0.1% TFA containing 6 M guanidine-HCl. These procedures allowed us to obtain soluble protein that was injected into the HPLC column. Protein pB1 was eluted from the HPLC column after approximately 25 min (Fig. 2, fraction II). This fraction contained only one polypeptide corresponding to pB1, as confirmed by N-terminal amino acid sequencing.

It is known that the BSP proteins potentiate bovine epididymal sperm capacitation [9, 25, 26]. Our results show that purified pB1 induces capacitation (as determined by induction of acrosome reaction by ionophore A23187; Fig. 5A). Interestingly, preincubation of sperm with 20 μg/ml or higher quantities (data not shown) of pB1 had no effect on epididymal sperm capacitation, while 10 μg/ml of pB1 induced

maximal capacitation. Similar effects have been observed previously with BSP proteins [26]. The reason that these proteins do no stimulate capacitation at higher concentrations is not known, but may relate to protein aggregation phenomena common to all BSP protein homologs [1, 2, 36, 41].

Interestingly, 10 μg/ml pB1 did not potentiate sperm capacitation in NCM (data not shown). NCM did not contain calcium or bicarbonate, constituents deemed important for boar sperm capacitation, which are probably required by pB1 in order to induce sperm capacitation.

Addition of boar seminal plasma to ejaculated sperm prevents their capacitation [53, 65, 66]. In the present study, the preincubation of boar epididymal sperm with different volumes of whole boar seminal plasma, corresponding approximately to the same concentration of purified pB1 used for capacitation experiments, did not induce capacitation (Fig. 4A). It is possible that seminal plasma contains factors that inhibit sperm capacitation. In vivo, sperm is not exposed to seminal plasma for a long period before coming in contact with female genital tract fluids [67]. The factors present in seminal plasma that inhibit sperm capacitation are probably released when sperm are in the female genital tract. In our studies, sperm were incubated in CM, and our media are probably not sufficient to remove these inhibiting factors to allow pB1 to induce capacitation.

The pB1 protein can bind the sperm membrane during preincubation. It was previously demonstrated that biotinylated pB1 protein could bind epididymal sperm immobilized in microtiter plates [42]. Our results show that after 4 h incubation in CM, less pB1 was bound to sperm as compared to the start of the incubation (Fig. 7). These results are similar to those observed with bovine sperm capacitation in which BSP-A1/-A2 proteins are removed from the sperm membrane following capacitation [27]. The bands with higher molecular weight (Fig. 7) correspond to nonspecific bands, because they are present in protein extracts of sperm preincubated without pB1.

The effect of BSP proteins on the capacitation of sperm from other species has not been studied to date. In the current study, preincubation of boar epididymal sperm with BSP-A1/-A2 potentiated capacitation (Fig. 6A). However, to induce capacitation at the same level, twice the amount of BSP-A1/-A2 was required as compared to pB1 (Figs. 5A and 6A). This discrepancy could be attributed to structural differences between the two proteins that could affect their binding affinity for the choline groups of the sperm membrane phospholipids. The amino acid sequences of BSP-A1/-A2 and pB1 share 65% similarity. It is also possible that the differences in glycosylation or aggregation status could affect the potency of these proteins in stimulating capacitation.

Based on the current study, we propose that the mechanism of boar sperm capacitation involving pB1 is similar to that occurring in the bovine species with BSP proteins. At ejaculation, epididymal boar sperm is mixed with seminal plasma, and pB1 present in seminal plasma binds to the sperm membrane. The effect of pB1 binding to the sperm membrane is unknown; however, as demonstrated with BSP proteins, pB1 could mediate cholesterol and phospholipid efflux or membrane destabilization. HCO_3^- has been shown to be important for the reorganization of the membrane lipid architecture of boar sperm [45, 46] and for sperm capacitation [68]. These studies were done with ejaculated sperm that were already in contact with pB1. It is possible that pB1 acts in synergy with HCO_3^- to reorganize the boar sperm membrane, activating unknown intracellular pathways that lead to capacitation.

Since BSP proteins potentiate sperm capacitation by GAGs and HDL, it would be interesting to determine whether heparin-like GAGs and HDL from the porcine oviductal fluid could act in synergy with pB1 to induce boar sperm capacitation. Since there is no heparin or GAGs in our capacitation media, it is possible that pB1 and BSP-A1/-A2 induce sperm capacitation via stimulation of a cholesterol and phospholipid efflux from the sperm membrane. These hypotheses need to be verified. In addition, pB1 could be implicated in the formation of a boar sperm reservoir in the female genital tract, as was shown for BSP proteins that promote bull sperm binding to oviductal epithelium [29, 30]. In fact, protein aggregates of boar seminal plasma that contain pB1 have been shown to interact strongly with the oviductal cells [69], suggesting the involvement of pB1 in the formation of the boar sperm reservoir. Thus, more studies are warranted to understand how pB1 capacitates boar sperm and allows sperm binding to the oviduct.

In summary, pB1 can be isolated and purified efficiently from boar seminal plasma by the new method described here. We are the first to demonstrate that pB1 could potentiate the capacitation of boar epididymal sperm. Also, boar epididymal sperm can be capacitated by the homologous bovine protein BSP-A1/-A2. The current study shows, for the first time, that BSP proteins homologs can induce the capacitation of epididymal sperm from another species. These results suggest that proteins of the BSP family and their homologs might play similar biological roles. Further investigations on the mechanism by which pB1 induces boar epididymal sperm capacitation are necessary. The understanding of boar sperm capacitation would aid the improvement of artificial insemination methods in this species, and would help to elaborate a general mechanism of mammalian sperm capacitation.

REFERENCES

1. Manjunath P, Sairam MR. Purification and biochemical characterization of three major acidic proteins (BSP-A1, BSP-A2 and BSP-A3) from bovine seminal plasma. Biochem J 1987; 241:685–692.
2. Manjunath P, Baillargeon L, Marcel YL, Seidah NG, Chrétien M, Chapdelaine A. Diversity of novel proteins in gonadal fluids. In: Mckerns KW, Chrétien M (eds.), Molecular Biology of Brain and Endocrine Peptidergic Systems. New York: Plenum Press; 1988:259–273.
3. Esch FS, Ling NC, Bohlen P, Ying SY, Guillemin R. Primary structure of PDC-109, a major protein constituent of bovine seminal plasma. Biochem Biophys Res Commun 1983; 113:861–867.
4. Seidah NG, Manjunath P, Rochemont J, Sairam MR, Chretien M. Complete amino acid sequence of BSP-A3 from bovine seminal plasma. Homology to PDC-109 and to the collagen-binding domain of fibronectin. Biochem J 1987; 243:195–203.
5. Calvete JJ, Mann K, Sanz L, Raida M, Topfer-Petersen E. The primary structure of BSP-30K, a major lipid-, gelatin-, and heparin-binding glycoprotein of bovine seminal plasma. FEBS Lett 1996; 399:147–152.
6. Desnoyers L, Manjunath P. Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. J Biol Chem 1992; 267: 10149–10155.
7. Manjunath P, Marcel YL, Uma J, Seidah NG, Chretien M, Chapdelaine A. Apolipoprotein A-I binds to a family of bovine seminal plasma proteins. J Biol Chem 1989; 264:16853–16857.
8. Chandonnet L, Roberts KD, Chapdelaine A, Manjunath P. Identification of heparin-binding proteins in bovine seminal plasma. Mol Reprod Dev 1990; 26:313–318.
9. Therien I, Bergeron A, Bousquet D, Manjunath P. Isolation and characterization of glycosaminoglycans from bovine follicular fluid and their effect on sperm capacitation. Mol Reprod Dev 2005; 71:97–106.
10. Manjunath P, Sairam MR, Uma J. Purification of four gelatin-binding proteins from bovine seminal plasma by affinity chromatography. Biosci Rep 1987; 7:231–238.
11. Manjunath P, Therien I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 2002; 53:109–119.
12. Austin CR. Observations on the penetration of the sperm in the mammalian egg. Aust J Sci Res (B) 1951; 4:581–596.

13. Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 1951; 168:697–698.
14. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd ed. New York: Raven Press; 1994: 189–317.
15. Therien I, Moreau R, Manjunath P. Major proteins of bovine seminal plasma and high-density lipoprotein induce cholesterol efflux from epididymal sperm. Biol Reprod 1998; 59:768–776.
16. Travis AJ, Kopf GS. The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. J Clin Invest 2002; 110: 731–736.
17. Handrow RR, Lenz RW, Ax RL. Structural comparisons among glycosaminoglycans to promote an acrosome reaction in bovine spermatozoa. Biochem Biophys Res Commun 1982; 107:1326–1332.
18. Lenz RW, Ax RL, Grimek HJ, First NL. Proteoglycan from bovine follicular fluid enhances an acrosome reaction in bovine spermatozoa. Biochem Biophys Res Commun 1982; 106:1092–1098.
19. Lenz RW, Ball GD, Lohse JK, First NL, Ax RL. Chondroitin sulfate facilitates an acrosome reaction in bovine spermatozoa as evidenced by light microscopy, electron microscopy and in vitro fertilization. Biol Reprod 1983; 28:683–690.
20. Meizel S, Turner KO. Glycosaminoglycans stimulate the acrosome reaction of previously capacitated hamster sperm. J Exp Zool 1986; 237:137–139.
21. First NL, Parrish JJ. In-vitro fertilization of ruminants. J Reprod Fertil Suppl 1987; 34:151–165.
22. Brantmeier SA, Grummer RR, Ax RL. Concentrations of high density lipoproteins vary among follicular sizes in the bovine. J Dairy Sci 1987; 70:2145–2149.
23. Ehrenwald E, Foote RH, Parks JE. Bovine oviductal fluid components and their potential role in sperm cholesterol efflux. Mol Reprod Dev 1990; 25: 195–204.
24. Jaspard B, Collet X, Barbaras R, Manent J, Vieu C, Parinaud J, Chap H, Perret B. Biochemical characterization of pre-beta 1 high-density lipoprotein from human ovarian follicular fluid: evidence for the presence of a lipid core. Biochemistry 1996; 35:1352–1357.
25. Therien I, Soubeyrand S, Manjunath P. Major proteins of bovine seminal plasma modulate sperm capacitation by high-density lipoprotein. Biol Reprod 1997; 57:1080–1088.
26. Therien I, Bleau G, Manjunath P. Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. Biol Reprod 1995; 52:1372–1379.
27. Therien I, Bousquet D, Manjunath P. Effect of seminal phospholipid-binding proteins and follicular fluid on bovine sperm capacitation. Biol Reprod 2001; 65:41–51.
28. Lane M, Therien I, Moreau R, Manjunath P. Heparin and high-density lipoprotein mediate bovine sperm capacitation by different mechanisms. Biol Reprod 1999; 60:169–175.
29. Gwathmey TM, Ignotz GG, Suarez SS. PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium in vitro and may be involved in forming the oviductal sperm reservoir. Biol Reprod 2003; 69:809–815.
30. Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. Biol Reprod 2006; 75: 501–507.
31. Calvete JJ, Mann K, Schafer W, Sanz L, Reinert M, Nessau S, Raida M, Topfer-Petersen E. Amino acid sequence of HSP-1, a major protein of stallion seminal plasma: effect of glycosylation on its heparin- and gelatin-binding capabilities. Biochem J 1995; 310:615–622.
32. Menard M, Nauc V, Lazure C, Vaillancourt D, Manjunath P. Novel purification method for mammalian seminal plasma phospholipid-binding proteins reveals the presence of a novel member of this family of protein in stallion seminal fluid. Mol Reprod Dev 2003; 66:349–357.
33. Villemure M, Lazure C, Manjunath P. Isolation and characterization of gelatin-binding proteins from goat seminal plasma. Reprod Biol Endocrinol 2003; 1:39.
34. Boisvert M, Bergeron A, Lazure C, Manjunath P. Isolation and characterization of gelatin-binding bison seminal vesicle secretory proteins. Biol Reprod 2004; 70:656–661.
35. Bergeron A, Villemure M, Lazure C, Manjunath P. Isolation and characterization of the major proteins of ram seminal plasma. Mol Reprod Dev 2005; 71:461–470.
36. Calvete JJ, Raida M, Gentzel M, Urbanke C, Sanz L, Topfer-Petersen E. Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma: primary structure of porcine pB1. FEBS Lett 1997; 407:201–206.
37. Leblond E, Desnoyers L, Manjunath P. Phosphorylcholine-binding proteins from the seminal fluids of different species share antigenic determinants with the major proteins of bovine seminal plasma. Mol Reprod Dev 1993; 34:443–449.
38. Fan J, Lefebvre J, Manjunath P. Bovine seminal plasma proteins and their relatives: a new expanding superfamily in mammals. Gene 2006; 375:63–74.
39. Plucienniczak G, Jagiello A, Plucienniczak A, Holody D, Strzezek J. Cloning of complementary DNA encoding the pB1 component of the 54-kilodalton glycoprotein of boar seminal plasma. Mol Reprod Dev 1999; 52:303–309.
40. Bezouska K, Sklenar J, Novak P, Halada P, Havlicek V, Kraus M, Ticha M, Jonakova V. Determination of the complete covalent structure of the major glycoform of DQH sperm surface protein, a novel trypsin-resistant boar seminal plasma O-glycoprotein related to pB1 protein. Protein Sci 1999; 8:1551–1556.
41. Jonakova VV, Manaskova P, Kraus M, Liberda J, Ticha M. Sperm surface proteins in mammalian fertilization. Mol Reprod Dev 2000; 56:275–277.
42. Jonakova V, Kraus M, Veselsky L, Cechova D, Bezouska K, Ticha M. Spermadhesins of the AQN and AWN families, DQH sperm surface protein and HNK protein in the heparin-binding fraction of boar seminal plasma. J Reprod Fertil 1998; 114:25–34.
43. Okamura N, Tanba M, Fukuda A, Sugita Y, Nagai T. Forskolin stimulates porcine sperm capacitation by increasing calcium uptake. FEBS Lett 1993; 316:283–286.
44. Suzuki K, Mori T, Shimizu H. In vitro fertilization of porcine oocytes in chemically defined medium. Theriogenology 1994; 42:1357–1368.
45. Harrison RA, Ashworth PJ, Miller NG. Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Mol Reprod Dev 1996; 45:378–391.
46. Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B, Gadella BM. Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. J Cell Sci 2001; 114:3543–3555.
47. Holt WV, Harrison RA. Bicarbonate stimulation of boar sperm motility via a protein kinase A-dependent pathway: between-cell and between-ejaculate differences are not due to deficiencies in protein kinase A activation. J Androl 2002; 23:557–565.
48. Shadan S, James PS, Howes EA, Jones R. Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. Biol Reprod 2004; 71:253–265.
49. Kalab P, Peknicova J, Geussova G, Moos J. Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. Mol Reprod Dev 1998; 51:304–314.
50. Flesch FM, Colenbrander B, van Golde LM, Gadella BM. Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. Biochem Biophys Res Commun 1999; 262:787–792.
51. Tardif S, Sirard MA, Sullivan R, Bailey JL. Identification of capacitation-associated phosphoproteins in porcine sperm electroporated with ATP-gamma-(32)P. Mol Reprod Dev 1999; 54:292–302.
52. Kim NH, Day BN, Lim JG, Lee HT, Chung KS. Effects of oviductal fluid and heparin on fertility and characteristics of porcine spermatozoa. Zygote 1997; 5:61–65.
53. Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodriguez-Martinez H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. Int J Androl 2002; 25:84–93.
54. Gadella BM, Harrison RA. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. Development 2000; 127:2407–2420.
55. Medeiros CM, Parrish JJ. Changes in lectin binding to bovine sperm during heparin-induced capacitation. Mol Reprod Dev 1996; 44:525–532.
56. Dubé C, Tardif S, LeClerc P, Bailey JL. The importance of calcium in the appearance of p32, a boar sperm tyrosine phosphoprotein, during in vitro capacitation. J Androl 2003; 24:727–733.
57. Wang WH, Abeydeera LR, Fraser LR, Niwa K. Functional analysis using chlortetracycline fluorescence and in vitro fertilization of frozen-thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. J Reprod Fertil 1995; 104:305–313.
58. Nauc V, Manjunath P. Radioimmunoassays for bull seminal plasma proteins (BSP-A1/-A2, BSP-A3, and BSP-30-Kilodaltons), and their quantification in seminal plasma and sperm. Biol Reprod 2000; 63:1058–1066.
59. Dott HM, Foster GC. A technique for studying the morphology of mammalian spermatozoa which are eosinophilic in a differential 'life-dead' stain. J Reprod Fertil 1972; 29:443–445.

60. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
61. Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 1987; 262:10035–10038.
62. Basak A, Boudreault A, Chen A, Chretien M, Seidah NG, Lazure C. Application of the multiple antigenic peptides (MAP) strategy to the production of prohormone convertases antibodies: synthesis, characterization and use of 8-branched immunogenic peptides. J Pept Sci 1995; 1: 385–395.
63. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978; 87:206–210.
64. Setchell BP, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 1st ed. New York: Raven Press; 1988:753–836.
65. Harayama H, Magargee SF, Kunze E, Shidara O, Iwamoto E, Arikawa S, Miyake M, Kato S, Hammerstedt RH. Changes in epididymal protein antiagglutinin on ejaculated boar spermatozoa during capacitation in vitro. Reprod Fertil Dev 1999; 11:193–199.
66. Vadnais ML, Kirkwood RN, Tempelman RJ, Sprecher DJ, Chou K. Effect of cooling and seminal plasma on the capacitation status of fresh boar sperm as determined using chlortetracycline assay. Anim Reprod Sci 2005; 87:121–132.
67. Williams-Ashman HG. Perspectives in the male sexual physiology of eutherian mammals. In: Knobil E, Neill JD, (eds.), The Physiology of Reproduction, 1st ed. New York: Raven Press; 1988:727–751.
68. Tardif S, Dubé C, Bailey JL. Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. Biol Reprod 2003; 68:207–213.
69. Liberda J, Manaskova P, Prelovska L, Ticha M, Jonakova V. Saccharide-mediated interactions of boar sperm surface proteins with components of the porcine oviduct. J Reprod Immunol 2006; 71:112–125.