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Isolation and characterization of gelatin-binding proteins from goat seminal plasma

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Abstract

A family of proteins designated BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa (collectively called BSP proteins for Bovine Seminal Plasma proteins) constitute the major protein fraction in the bull seminal plasma. These proteins interact with choline phospholipids on the sperm surface and play a role in the membrane stabilization (decapacitation) and destabilization (capacitation) process. Homologous proteins have been isolated from boar and stallion seminal plasma. In the current study we report the isolation and preliminary characterization of homologous proteins from goat seminal plasma. Frozen semen (-80°C) was thawed and centrifuged to remove sperm. The proteins in the supernatant were precipitated by the addition of cold ethanol. The precipitates were dissolved in ammonium bicarbonate and lyophilised. The lyophilised proteins were dissolved in phosphate buffer and loaded onto a gelatin-agarose column, which was previously equilibrated with the same buffer. The column was successively washed with phosphate buffer, with phosphate buffer saline and with 0.5 M urea in phosphate buffer saline to remove unadsorbed proteins, and the adsorbed proteins were eluted with 5 M urea in phosphate buffer saline. Analysis of pooled, dialysed and lyophilised gelatin-agarose adsorbed protein fraction by SDS-PAGE indicated the presence of four protein bands that were designated GSP-14 kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa (GSP, Goat Seminal Plasma proteins). Heparin-affinity chromatography was then used for the separation of GSP-20 and -22 kDa from GSP-14 and -15 kDa. Finally, HPLC separation permitted further isolation of each one from the other. Amino acid sequence analysis of these proteins indicated that they are homologous to BSP proteins. In addition, these BSP homologs bind to hen's egg-yolk low-density lipoproteins. These results together with our previous data indicate that BSP family proteins are ubiquitous in mammalian seminal plasma, exist in several forms in each species and possibly play a common biological role.

Background

The major acidic proteins contained in bovine seminal plasma represent a family called BSP (Bovine Seminal

Plasma) proteins [1,2]. They are designated BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa. The latter is named after its molecular mass, while BSP-A1, -A2 and -A3 have

molecular masses between 15 and 17 kDa. BSP-A1 and BSP-A2 differ only by the carbohydrates they contain, therefore they are considered glycoforms of the same protein, named BSP-A1/-A2, and also known as PDC-109 [3]. Homologous proteins have been found in boar (pB1; [4,5]) and stallion (HSP-1, HSP-2; [5,6]). BSP-like antigens have also been detected in rat, mouse, hamster, and human [7].

The biological properties of BSPs have been extensively studied [8]. Their role in fertilization, specifically in sperm capacitation, is conferred by their binding to the choline group of phospholipids present in the sperm membrane [9], and to high-density lipoproteins (HDL) and heparin-like glycosaminoglycans (GAGs) present in the follicular and oviductal fluids [10–12]. The general mechanism of capacitation that is proposed by our laboratory includes continuous and progressive modification of sperm membrane by BSP proteins (reviewed in [8]). At ejaculation, sperm are mixed with seminal fluid or BSP proteins (secreted by accessory glands) for a brief period (10–15 min). During this brief exposure BSP proteins remove some (5–8%) cholesterol (1st cholesterol efflux) from the sperm membrane. At the same time BSP proteins bind to choline phospholipids composed of sperm plasma membrane. This prevents the free movement of phospholipids and stabilizes the sperm membrane. As the sperm reach the oviduct, sperm-bound BSP proteins interact with the HDL that is present in the oviduct and/or follicular fluids. This results in further removal of cholesterol (2nd cholesterol efflux) as well as removal of BSP proteins from the sperm membrane [12]. This destabilizes the membrane and induces some intracellular signal transduction pathways, among which the increase in its membrane permeability to Ca²⁺ and in its intracellular pH. Heparin-like GAGs in the female genital tract could also play a role in this capacitation mechanism, since they also interact with BSPs.

Conversely to this positive role in fertility, BSP proteins may also have negative effects in the context of sperm storage. It is shown that the cholesterol efflux induced by BSP proteins is time and concentration dependant, thus too long exposure of sperm to these proteins, or exposure to too large concentrations of them, could be deleterious to the sperm membrane. Our recent results indicate that BSP proteins form stable complexes with low-density lipoproteins of hen's egg-yolk used in diluters or extenders (sperm preservation media). Consequently, the deleterious effect of BSP proteins on sperm is either minimized or completely inhibited, and hence sperm can be stored in liquid or frozen state [13].

To date, the identity and biological role of the goat seminal plasma protein components have not been studied,

though the presence of heparin-affinity proteins (HAPs) have been reported [14]. The structure of the BSPs show two type II domains (or fibronectin gelatin binding domains) arranged in tandem fashion [2,3,15–17]. These domains interact with collagen and with its denatured derivative, gelatin. We used this binding property to isolate BSP-like homologs from goat seminal plasma, which was the main objective of this investigation. We also obtained N-terminal sequence of those proteins and tested their binding properties to heparin and low-density lipoprotein fraction (LDF), in order to confirm their structure-function relationship to BSPs.

Materials and Methods

Materials

Gelatin was purchased from Eastman Kodak Company (Rochester, New-York, USA). Affigel-15 was from Bio-Rad (Mississauga, ON, Canada) and Sephadex G-50 (super fine) and heparin-Sepharose CL-6B from Amersham Biosciences (Baie d'Urfé, QC, Canada). Acrylamide and bisacrylamide were purchased from ICN (Mississauga, ON, Canada). Sodium dodecyl sulfate (SDS) and other electrophoresis products were from Bio-Rad. Immobilon-P polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Nepean, ON, Canada). HPLC grade trifluoroacetic acid (TFA) was from Fisher Scientific (St-Laurent, QC, Canada). All other chemicals used were of ultra pure grade and obtained from local suppliers, mostly Fisher Scientific and Sigma-Aldrich (Oakville, ON, Canada). Goat semen was provided by the Centre d'Insemination Ovine du Quebec.

Methods

Isolation of the seminal plasma proteins

Ten ml of frozen semen (-80°C) was thawed and centrifuged at 1 000 × g for 10 min to remove sperm. The supernatant was further centrifuged at 10 000 × g for 10 min to obtain clear seminal plasma. Nine volumes of cold ethanol were added and left with stirring for 90 min at 4°C to precipitate the proteins, that were then recovered by centrifugation at 10 000 × g for 10 min. After three subsequent ethanol washes, the precipitates were solubilized in 50 mM ammonium bicarbonate and lyophilised. Approximately 270 mg of dried powder was recovered. The protein content of the powder was determined using the modified Lowry's method [18].

Gelatin-agarose affinity chromatography

Gelatin was previously coupled to Affi-Gel 15 as described earlier [2]. All purification steps were conducted at 4°C. Lyophilised proteins were dissolved in phosphate buffer (PB) and loaded on a gelatin-agarose column, which was previously equilibrated with the same buffer. Once the sample entered the column, the flow was stopped for 15 min to allow proteins to bind. After contaminants and

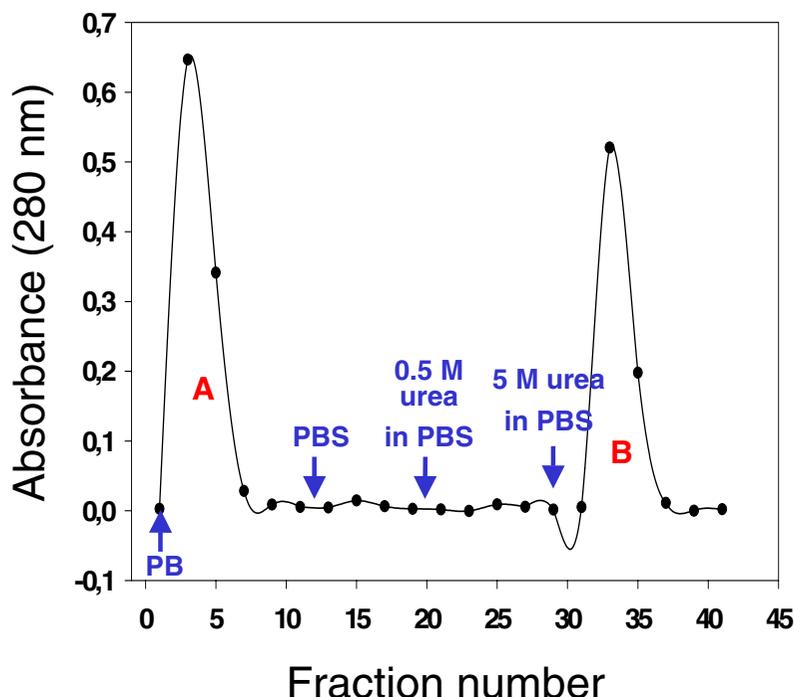


Figure 1

Gelatine-agarose affinity profile of goat seminal plasma proteins. Ten mg of goat seminal plasma proteins were dissolved in 2 ml of PB and loaded on the column (1 × 20 cm). The unadsorbed proteins were removed by washing successively with PB, PBS and 0.5 M urea in PBS. Five M urea in PBS was then used to elute the gelatin-binding proteins. Four ml fractions were collected at a flow rate of 30 ml/h and pooled as indicated (fraction A and B). Proteins were monitored at 280 nm.

unadsorbed proteins were washed successively with PB, phosphate buffer saline (PBS) and 0.5 M urea in PBS, the bound proteins were eluted with a solution of 5 M urea in PBS. Protein-containing fractions were collected and pooled (Fig. 1). Fraction A was dialysed against 50 mM ammonium-bicarbonate and lyophilised; whereas Fraction B was used in the next step.

Heparin-Sepharose affinity chromatography

The following steps were conducted at 4 °C at a flow rate of 20 ml/h. The gelatine-agarose adsorbed proteins (Fraction B) were dialysed against PB, concentrated to 2 ml and loaded on a heparin-Sepharose CL-6B column, which was previously equilibrated with the same buffer. Once the sample entered the column, the flow was stopped for 15

min to allow proteins to bind. The column was washed with PB to remove unadsorbed proteins and the bound proteins were eluted with 1 M NaCl in PB. Four ml fractions were collected and their absorbance at 280 nm was measured. They were pooled and then dialysed against 50 mM ammoniumbicarbonate and lyophilised.

SDS-PAGE and blotting

SDS-PAGE was performed according to Leammli [19] on 10% or 15% polyacrylamide gels, using the Mini protein 3 apparatus from Bio-Rad. Molecular mass was estimated by comparison with the Low Molecular Weight Calibration Kit from Amersham Biosciences. Proteins from the SDS-PAGE were then either stained with Coomassie Blue or transferred electrophoretically to Immobilon-P PVDF

membranes as described previously [20], using Trans-Blot Cell apparatus from Bio-Rad. Transferred proteins were stained with a 0.2% Ponceau S solution in 3% acetic acid for N-terminal sequence analysis.

RP-HPLC

Reversed phase-high performance liquid chromatography (RP-HPLC) was performed using a Vydac C18 column (250 × 0.4 mm, 5 µm, 300 Å; Mandel Scientific, St-Laurent, QC, Canada) connected to a Beckman Gold system (Beckman, Mississauga, ON, Canada). This system includes a 126 solvent module, a 168 detector equipped with a deuterium lamp, and a 32 Karat analysis software, and the external injector is from Rheodyne. Dialysed proteins from the gelatin-agarose chromatography, following lyophilization, were dissolved in 0.1% TFA and loaded on the column and separated using a gradient of acetonitrile in 0.1% TFA. The eluting proteins were monitored at 235 nm, collected in 500 µL fractions and dried under vacuum.

Sequencing

Following transfer of the proteins separated by SDS-electrophoresis to Immobilon-P membrane, the Ponceau-red stained protein bands were cut and placed in the sequenator reactor. Sequencing was carried out according to the manufacturer's protocol using an Applied Biosystems Pro-cise sequencer (model 494).

Interaction of goat seminal plasma proteins with hen's egg yolk lipoproteins

The binding of GSPs to Hen's egg yolk LDF, isolated on a KBr solution as described previously [13], was studied using Paragon electrophoresis kit from Beckman. LDF (5.6 µg) was incubated with fraction B2 containing GSP-20 and GSP-22 kDa (45, 67.5 and 90 µg), or fraction B1 containing GSP-14 and GSP-15 kDa (5.6 and 22.4 µg), in a total volume of 15 µL Tris-HCl buffer. After 15 min incubation, 4 µL of each sample were applied to Lipo gel (0.5% agarose) slots. The electrophoresis was run at 100 V for 30 min, the gel was dipped in fixative solution and dried. The lipid bands were subsequently stained in Sudan black B.

Results

Isolation and purification of gelatin-binding proteins

Figure 1 shows the gelatin-agarose chromatography pattern of proteins precipitated from goat seminal plasma. Column was washed to remove unadsorbed proteins (fraction A), and the adsorbed ones were eluted with 5 M urea (fraction B). The total weight recovered from this chromatography step was ~72%, and the adsorbed proteins constituted almost 50% of it. Each fraction was analyzed by SDS-PAGE (Figure 2), which revealed the presence of four different proteins of apparent molecular

masses 14 kDa, 15 kDa, 20 kDa and 22 kDa. We designated those Goat Seminal Plasma proteins, respectively GSP-14 kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa. We observed that the unadsorbed fraction A contained a certain amount of proteins with a molecular mass similar to the GSPs on SDS-PAGE, that did not bind the gelatin-agarose (Figure 2). It is not likely that those proteins are GSPs, but other proteins of same apparent molecular weight contained in seminal plasma, since they again didn't bind to gelatin-agarose after we rechromatographed the unadsorbed fraction on the column (data not shown). Alternatively, a certain amount of GSP proteins could be in aggregated form or associated with phospholipids and thus would not be able to bind the column.

Proteins from the gelatin-adsorbed fraction B (containing the four GSPs) were loaded on a heparin-Sepharose affinity column (Figure 3). After we washed the column from unadsorbed proteins with PB (fraction B1), the adsorbed ones were eluted in a very broad peak with a solution of 1 M NaCl in PB (fraction B2). Approximately, 85% of the starting material (fraction B) was recovered after this step. SDS-PAGE analysis showed that GSP-14 and GSP-15 kDa were contained in fraction B1, so they did not bind heparin (Figure 2). However, GSP-20 kDa and GSP-22 kDa have the property to bind heparin since they were contained in fraction B2.

We used HPLC in order to further separate each GSP from each other. A C18 column and a slow gradient permitted the recovery of the four different proteins eluting in distinct peaks, except for an overlap between the eluting peak of GSP-20 kDa (peak II) and GSP-15 kDa (peak III), when separation was done directly from fraction B, containing the four gelatin-adsorbed proteins (Figure 4a). Consequently, by using fractions B1 and fraction B2, generated by a previous heparin-affinity step, which isolated GSP-14 and -15 kDa from GSP-20 and -22 kDa, we were able to obtain clear separation (Figure 4b and 4c). The identity of each peak was determined by SDS-PAGE analysis (data not shown). Peak III and IV were quite sharp and homogeneous, whereas proteins contained in peak I and II appeared to be more heterogeneous. They also seemed to have less u.v. absorbance compared to peak III and IV in fraction A (Figure 4a), however when stained in Coomassie Blue they all seemed to bind same amount of dye.

Comparison of the GSP and BSP proteins sequence homology

N-terminal sequencing of the first few residues of goat seminal plasma proteins showed that GSP-14 kDa and GSP-15 kDa share a lot of similarity with each other, as well as GSP-20 kDa with GSP-22 kDa (Figure 5). In addition, they contain the characteristic sequence found in type II domains [21], particularly the motif CVFPFX(Y)(R/

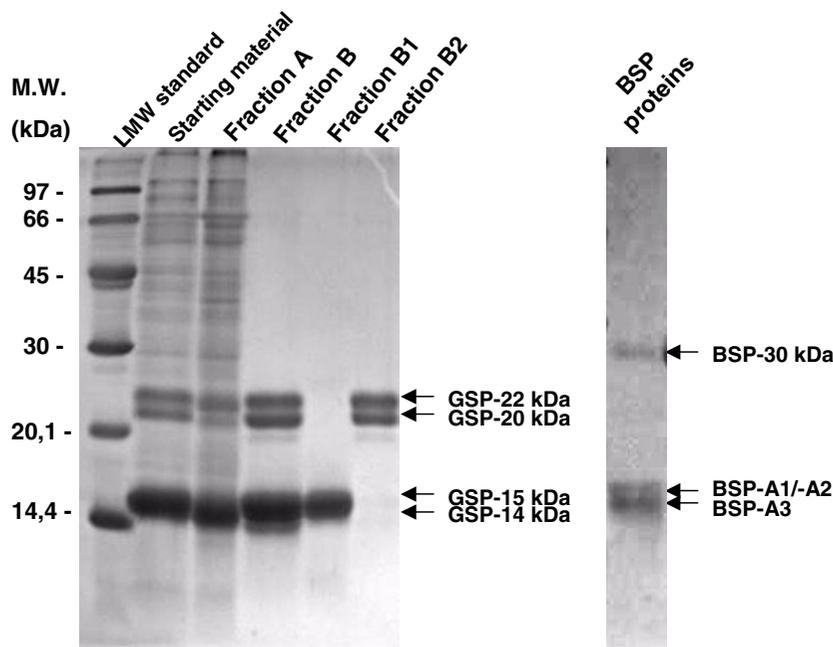


Figure 2
SDS-PAGE pattern of gelatin-agarose and heparin-Sepharose eluted fractions. Between 5 to 10 µg of lyophilised proteins from each fraction (except fraction A: 20 µg) were previously reduced and denatured, and loaded on a 15% gel. After migration of the proteins, the gel was stained with Coomassie brilliant Blue R-250. Electrophoresis pattern of the starting material is shown, as well as the pattern of BSP proteins for comparison.

K)X(R/K)(R/K)(H/Y)F that is much conserved among BSPs and other homologs found in boar and stallion seminal plasma proteins [22]. In GSP-20 kDa and GSP-22 kDa, the motif is not strictly respected, markedly the FPF part replaced by EAF, but the general pattern of amino acids is followed.

Interaction of GSP proteins with LDF

We tested gelatin adsorbed goat seminal plasma proteins for binding to LDF isolated from hen's egg-yolk. After they were incubated together, we submitted the samples to electrophoresis on lipogels (0.5% agarose gels). As seen on Figure 6, compared to the LDF alone (lane 2), LDF incubated with GSPs migrated towards the cathode (lanes 3-7), indicating that interactions were formed with the

GSP proteins, changing the overall charge of the molecule. It required four times higher concentrations of GSP-20 and -22 kDa than of GSP-14 and -15 kDa to produce this effect, nevertheless it demonstrates that all four proteins have the property to bind to LDF.

Discussion

In this study, we isolated the proteins from goat seminal plasma that are homologous to the BSP family of proteins from bovine. The method we describe contained few steps and was effective to obtain a good yield of pure proteins. We used a gelatin-agarose affinity column followed by heparin-Sepharose affinity column and HPLC separation, and we identified four proteins, that we named GSP-14 kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa, accord-

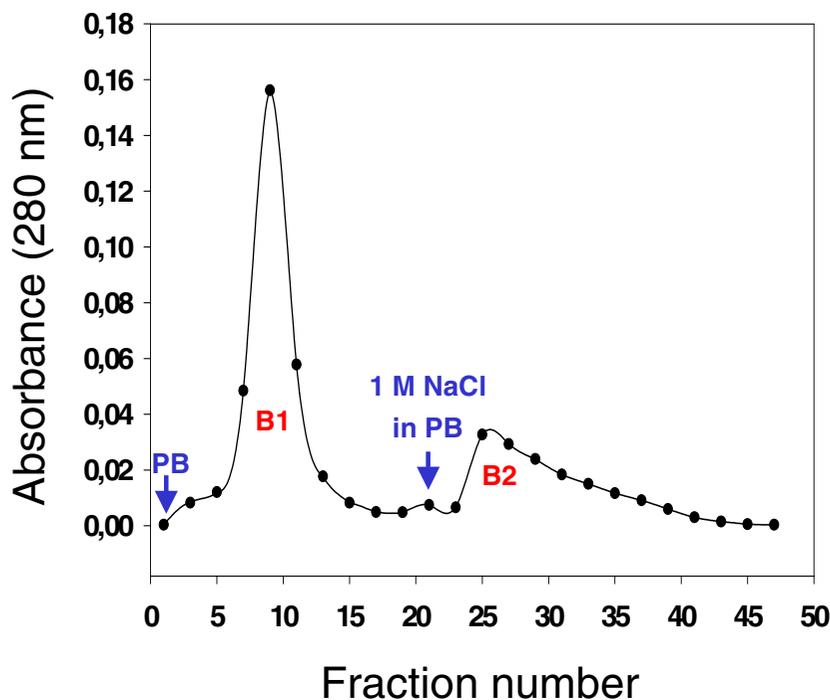


Figure 3

Heparin-Sepharose pattern of gelatin-adsorbed proteins. Lyophilised proteins from gelatin-agarose fraction B were resuspended in 2 ml PB and loaded on the column (1 × 13.5 cm). The unadsorbed proteins were removed by washing with PB, then 1 M NaCl in PB was used to elute the heparin-bound proteins. Four ml fractions were collected at a flow rate of 30 ml/h and pooled as indicated (fraction B1 and B2). Proteins were monitored at 280 nm.

ing to their apparent molecular weight. Together they constitute approximately 50% of the total proteins of goat seminal plasma. N-terminal sequencing of the first 30 to 50 residues of the GSP proteins confirmed that they are structurally related to BSPs as anticipated. Our studies also showed the ability of these proteins to bind lipoproteins.

A gelatin-agarose column was previously used to purify BSP proteins [2], given the type II domains that confer them the property to bind collagen, and thus gelatin, a denatured collagen. Subsequently, gelatin-binding capabilities of BSP protein homologs pB1 from boar, and HSP-1 and HSP-2 from stallion were also demonstrated [6,22]. Hence we can deduce that the GSPs we identified, by their gelatin binding properties, are also constituted of type II

structures. We also suggest they contain two such domains, in view of their molecular weight. Complete amino acid sequence, along with disulfide bridge assignment, should confirm their structural similarity.

The sequences obtained for the first 30 N-terminal amino acids of GSP-14 kDa and GSP-15 kDa differ only by few residues, and the same observation was made for the first 50 amino acids of GSP-20 kDa and GSP-22 kDa. Each of these pairs could be composed of quite similar proteins that may differ principally by the carbohydrates they contain. This would account for the fact that they migrate as "doublets" of protein bands on SDS-PAGE and show differences in their HPLC profiles. The same results were obtained in our lab for BSP-A1 and BSP-A2 when purified by

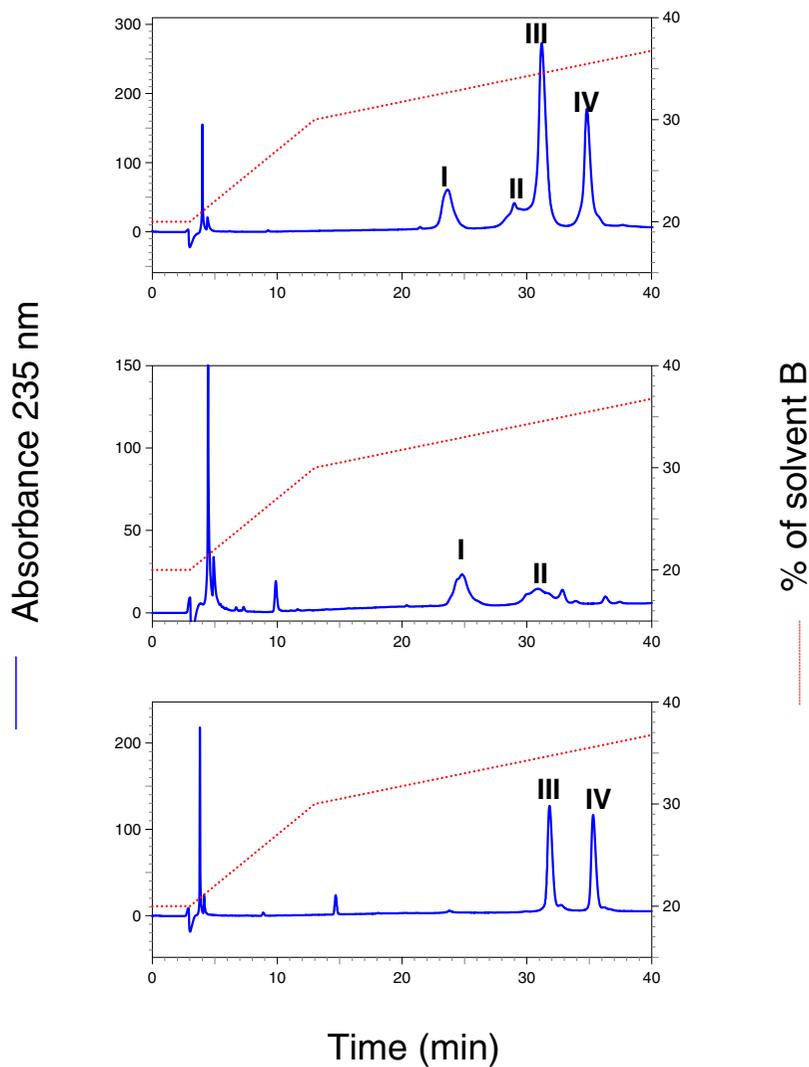


Figure 4

RP-HPLC pattern of gelatin-agarose and heparin-Sepharose eluted fractions. Approximately 100 μ g of proteins were dissolved in 1 ml of 0.1% (v/v) TFA in water and injected on a Vydac C18 column. They were eluted at 1 ml/min using a 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) buffer system: first isocratically at 20% B for 3 min, followed by a gradient of 20 to 30% B in 10 min, and 30 to 40% B in 40 min. **Top:** gelatin-agarose fraction B; **middle:** heparin-Sepharose fraction B2; **bottom:** heparin-Sepharose fraction B1. Peaks are identified as follow: I, GSP-22 kDa; II, GSP-20 kDa; III, GSP-15 kDa and IV, GSP-14 kDa.

| | 1 | 11 | 21 | 31 | 41 | 51 |
|-------------|------------------------|--------------------|--------------------|--------------------|------------------------|---------------------------------|
| GSP-20 kDa: | DE PPPYVYNL | PET PPPTSSY | YIP GFQVEKP | HE DDSXPRAF | AFT YK... | |
| GSP-22 kDa: | DE PPPYVYNL | PE M-PTSIPY | YS ADVQGP-- | -----P | XAF | AFT YRRKIYI Y... |
| GSP-14 kDa: | | | D | DEL TRDKS-E | ES HEDEAXVF | PFT YKDKKHF ... |
| GSP-15 kDa: | | | D | DEL TRDKSSE | ES HEEANXVF | PFT YKDK-HK ... |
| BSP-A1/A2: | ... LDP VNGDQ-- | -DE GVSTEPT | QD GPA---EL | PE --DEECVF | PF VYRNRKHF ... | |
| BSP-A3: | | DQ QL | SE DNVILPKE | KK DPA---SG | AET KDNKCVF | PFI YGNKKYF D ... |
| BSP-30 kDa: | ... LP RTIYPQEE | MP YDDKPFPS | LL SKANDLN- | AV FEGPACAF | PFT YKGGKYY | M ... |

Figure 5
N-terminal sequence comparison between GSP and BSP proteins. Residue numbering is indicated for GSP-20 and GSP-22 kDa, and the other sequences were aligned to show homology. The " - " indicates spaces left for alignment purposes, and "... " indicates continuation of the protein sequence at either end (N- or C-terminal). The identical amino acids are presented in bold.

gelatin-agarose [2]. Sequencing of the GSP proteins also indicate that they are homologous to BSP proteins, because they contain the specific motif of type II domains shared by all the BSPs and their homologs found in boar, stallion [6,22] and ram [Manjunath et al., unpublished data]. In Figure 4, X represents cysteine residues that have been turned to phenylthiohydantoin (PTH)-Cys derivative by the Edman's reagent and are thus not detectable. Under certain conditions other residues can also be destroyed during sequencing, but those especially are expected to be Cys because of their location among the other amino acids composing the characteristic sequence of type II domain.

BSP proteins are known to bind heparin and heparin-like glycosaminoglycans present in the female reproductive tract of mammals [23] and modulate capacitation via this interaction [10]. This interaction is made through stretches of basic amino acid residues resulting in a highly positive charge on the protein that counteracts the acidic groups of GAGs [24]. The BSP homologous proteins that have been found in boar (pB1) and stallion (HSP-1 and HSP-2) have all been found to also bind heparin [4-6]. GSPs were expected to have the same property. We found out that GSP-20 and GSP-22 kDa indeed bound to heparin, but GSP-14 and GSP-15 kDa did not. We used this heparin binding property to separate GSP-14 and -15 kDa from GSP-20 and -22 kDa. La Falci et al. [14], recently identified heparin-affinity proteins (HAPs) from goat

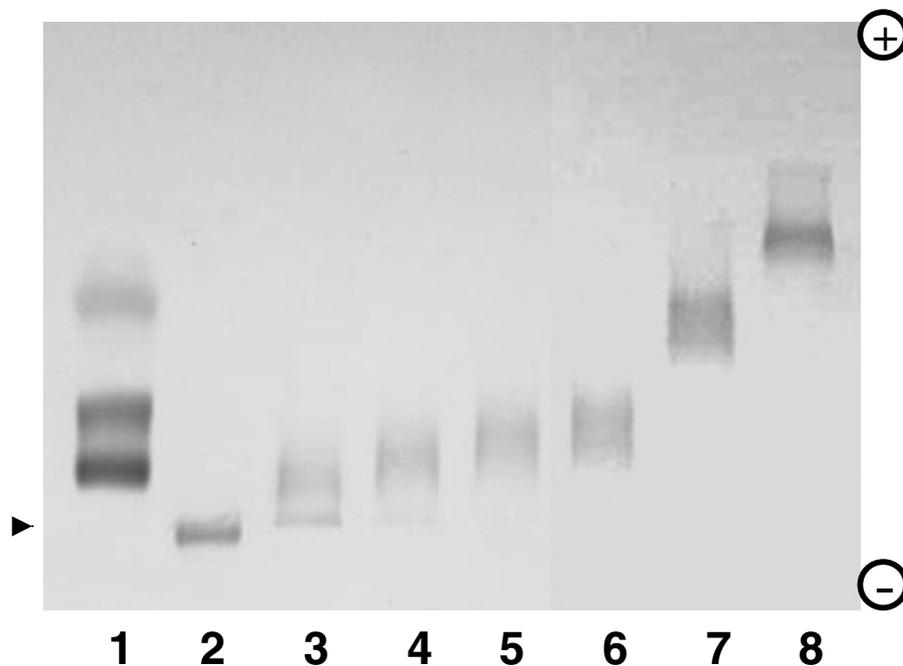


Figure 6

Electrophoretic analysis of the interaction of GSPs with LDF. Four μL of the samples (except the human serum used as a positive control of the migration) were applied on the lipogel. Lane 1: human serum (3 μl); lane 2: LDF (1.5 μg); lane 3, 4 and 5: LDF (1.5 μg) + fraction B2 containing GSP-20 kDa and GSP-22 kDa (12 μg , 18 μg , and 24 μg respectively); lane 6 and 7: LDF (1.5 μg) + fraction B1 containing GSP-14 kDa and GSP-15 kDa (1.5 μg and 6 μg , respectively); lane 8: LDF (1.5 μg) + BSP proteins (6 μg).

seminal plasma, however they were of molecular weights ranging from 73 to 104 kDa, and also of 119 and 178 kDa, but not from 20 to 22 kDa. Our investigation on alcohol precipitated proteins of goat seminal plasma indicate the presence of a group of proteins of apparent molecular masses between 73 and 95 kDa and a 119 kDa protein in heparin adsorbed material (data not shown), but GSP-20 kDa and GSP-22 kDa seem to be the major heparin binding proteins. The role of BSP family of proteins in capacitation is well established in bovine [8,12]. The availability of purified BSP homologs from stallion, boar and goat seminal plasma should facilitate similar studies on capacitation in these species. Although cross-reacting BSP homologs have been detected in human seminal fluid, they

have not been isolated and characterized to date. This is particularly because the human semen is not readily available and moreover, the quantity of BSP homologs in human seminal plasma is very low.

In conclusion, we have shown that goat seminal plasma contains a group of four proteins that are structurally related to the BSP family of proteins found in bull, boar and stallion. The GSP proteins show the same gelatin affinity and have the property to bind to LDF. However, only GSP-20 and GSP-22 kDa bind to heparin, while GSP-14 kDa and GSP-15 kDa do not. Their N-terminal sequences also encompass the characteristic amino acid composition of type II domains. These results clearly demonstrate that

proteins of the BSP family are ubiquitous in mammals and are meant to play a similar biological role. Since not much is known on the molecular events of capacitation of goat sperm and seminal plasma factors that affect goat sperm storage, the identification and characterization of proteins involved in these processes would aid further understanding of reproductive mechanisms.

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