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Ion-exchange chromatography used to isolate a spermadhesin-related protein from domestic goat (*Capra hircus*) seminal plasma

Dárcio Ítalo Alves Teixeira[1], Luciana Magalhães Melo[2], Carlos Alberto de Almeida Gadelha[2], Rodrigo Maranguape Silva da Cunha[2], Carlos Bloch Jr.[3], Gandhi Rádis-Baptista[2], Benildo Sousa Cavada[2] and Vicente José de Figueirêdo Freitas[1]

[1]Laboratório de Fisiologia e Controle da Reprodução, Universidade Estadual do Ceará, Fortaleza, CE, Brasil
[2]BioMol-Lab, Universidade Federal do Ceará, Fortaleza, CE, Brasil
[3]Laboratório de Espectrometria de Massa, EMBRAPA-CENARGEN, Caixa Postal 02372, Brasília, DF, Brasil
Corresponding author: V.J.F. Freitas
E-mail: vjff@uece.br

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ABSTRACT. Mammalian seminal plasma contains among others, proteins called spermadhesins, which are the major proteins of boar and stallion seminal plasma. These proteins appear to be involved in capacitation and sperm-egg interaction. Previously, we reported the presence of a protein related to spermadhesins in goat seminal plasma. In the present study, we have further characterized this protein, and we propose ion-exchange chromatography to isolate this seminal protein. Semen was obtained from four adult Saanen bucks. Seminal plasma was pooled, dialyzed against distilled water and freeze-dried. Lyophilized proteins were loaded onto an ion-exchange chromatography column. Dialyzed-lyophilized proteins from the main peak of DEAE-Sephacel were applied to a C2/C18 column coupled to an RP-HPLC system, and the eluted proteins were lyophilized for electrophoresis. The N-terminal was sequenced and amino acid sequence similarity was determined using

CLUSTAL W. Additionally, proteins from DEAE-Sephacel chromatography step were dialyzed and submitted to a heparin-Sepharose high-performance liquid chromatography. Goat seminal plasma after ion-exchange chromatography yielded 6.47 ± 0.63 mg (mean ± SEM) of the major retained fraction. The protein was designated BSFP (buck seminal fluid protein). BSFP exhibited N-terminal sequence homology to boar, stallion and bull spermadhesins. BSFP showed no heparin-binding capabilities. These results together with our previous data indicate that goat seminal plasma contains a protein that is structurally related to proteins of the spermadhesin family. Finally, this protein can be efficiently isolated by ion-exchange and reverse-phase chromatography.

Key words: Goat, Seminal plasma, Lectin, Spermadhesin

INTRODUCTION

Stockert et al. (1974) published a paper describing an agglutinin for human or lapine erythrocytes found in rabbit liver. This "mammalian hepatic lectin", as they called it, was claimed to be "the first lectin of mammalian origin". Animal lectins mediate biological processes ranging from protein folding and trafficking to the modulation of cell-cell and cell-matrix interactions (Drickamer and Taylor, 1993; Gabius, 1997).

Seminal plasma, the fluid in which mammalian spermatozoa are suspended in semen, is a complex mixture of secretions originating from the epididymis and the male accessory reproductive organs. It contains a variety of both inorganic and organic components, among which proteins are a major part of the high-molecular mass substances. The protein composition of seminal plasma varies from species to species, but studies on several mammalian species indicate that seminal plasma contains factors that influence the fertilizing ability of spermatozoa and exert important effects on female reproductive physiology (Shivaji et al., 1990; Waberski et al., 1995).

A protein family called spermadhesins has been detected in seminal plasma of many domestic species. These proteins are a group of 12- to 16-kDa polypeptides found in the seminal plasma and peripherally associated with the spermatozoa surface. They are multifunctional proteins exhibiting ligand-binding affinities to an array of oligosaccharides and sulfated polysaccharides such as heparin and also to serine protease inhibitor and phospholipids (Töpfer-Petersen et al., 1998). The amino acid sequence of these proteins does not show any discernible similarity to known carbohydrate-recognition domains, indicating that spermadhesins may belong to a novel group of animal lectins.

Boar spermadhesins are a group of proteins found in seminal plasma, and they are major secretory products of the seminal vesicle epithelium. These proteins are composed of 109-133 amino acids, contain two conserved disulfide bridges, and have 40-60% amino acid sequence identity (reviewed in Töpfer-Petersen et al., 1995, 1998). Both non- and *N*-glycosylated forms of spermadhesins have been described (Calvete et al., 1993). Two spermadhesin subfamilies AQN (AQN-1, AQN-2, and AQN-3) and AWN (AWN-1, and AWN-2) - named

according to their first three N-terminal amino acids - were shown to bind to the acrosomal cap of boar epididymal spermatozoa, which classified them as sperm surface proteins (Jonakova et al., 1998). The affinity of sperm-bound spermadhesins for acid polysaccharides pointed to their interaction with heparin-like glycosaminoglycans secreted by the epithelium of the female reproductive tract (Tienthai et al., 2000). This hypothesis led to the idea that these proteins are involved in sperm capacitation. Besides that, their ability to bind to zona pellucida glycoproteins has suggested their role in sperm-egg interactions (Sanz et al., 1993; Jonakova et al., 1998; Ticha et al., 1998).

Proteins homologous to boar spermadhesins were also found in stallion (Calvete et al., 1995a) and bull (Einspanier et al., 1994; Calvete et al., 1996) seminal plasma. The study of homologous proteins in seminal plasma of different species may elucidate their functions in the fertilization process.

In our previous communication, we reported the presence of a low-molecular mass protein related to boar spermadhesins in goat seminal plasma (Teixeira et al., 2002). In the present study, we characterized this protein further, and we propose ion-exchange chromatography as a method to best isolate this seminal protein.

MATERIAL AND METHODS

Local and experimental animals

This experiment was carried out at both the Laboratory of Physiology and Control of Reproduction (State University of Ceará) and Biomol-Lab (Federal University of Ceará), located in Fortaleza, CE, Brazil (3°43'S, 38°30'W).

Four Saanen bucks (*Capra hircus*) used for semen collection were aged 2 to 4 years and individually penned (4 m^2/pen) in a well-ventilated shed. The animals were fed with elephant grass (*Pennisetum purpureum*) and with a concentrate mixture (18% crude protein minimum). They had free access to water and mineral blocks.

Collection and storage of semen

Semen was collected twice a week always at 7:00 am with the use of an artificial vagina. The collection procedure was facilitated by the use of a female goat that had been estrus-induced by an intramuscular injection of estradiol. After collection, the semen was evaluated for the following parameters: volume, mass motility and progressive individual motility, centrifuged at 800 *g* for 10 min, and the sperm pellet was discarded. The supernatant (seminal plasma) was stored at -20°C until be used.

Isolation

Pooled seminal plasma was dialyzed against distilled water and freeze-dried. Lyophilized proteins (20 mg) were dissolved in 0.02 M phosphate buffer, pH 7.0, and loaded onto an ion-exchange chromatography column (DEAE-Sephacel, 1.2 x 20 cm), which was previously equilibrated with the same buffer. After removal of the non-retained material, the column was eluted with a NaCl gradient (0-1 M) in buffer.

Dialyzed-lyophilized proteins from the main peak of DEAE-Sephacel were applied to a C2/C18 column (100 x 4.6 mm, 3 µm, 120 Å; Amersham Biosciences, Uppsala, Sweden) coupled to a reverse phase-high-performance liquid chromatography (RP-HPLC) system. They were eluted using 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) buffer system: first isocratically at 0% B (7 min), followed by a gradient of 0-40% B (4 min), 40-45% B (11 min), and 100% B (10 min). Fractions were collected at a flow rate of 1 mL/min and monitored at 280 nm. Eluted proteins were lyophilized for electrophoresis.

Detection and characterization

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% polyacrylamide gels according to Laemmli (1970), and proteins were silver-stained. The N-terminal of putative spermadhesin was sequenced on an Applied Biosystems 477A (Applied Biosystems, Foster City, CA, USA).

Proteins from the DEAE-Sephacel chromatography step were dialyzed against 0.02 M phosphate buffer, pH 7.0, concentrated to 0.5 mL and loaded onto a heparin-Sepharose high-performance column (0.7 x 2.5 cm, 34 µm; Amersham Biosciences), 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 removal of the non-retained material, the column was eluted with an NaCl gradient (0-1 M) in buffer. Fractions were collected at a flow rate of 1 mL/min and monitored at 280 nm. Eluted proteins were lyophilized for SDS-PAGE, as described above.

Multiple alignment for similarity search

Amino acid sequence similarity searches were carried out using the program CLUSTAL W (Chenna et al., 2003). A cladogram tree was made using the same program and according to the PHYLIP method (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Although several seminal plasma proteins of different mammalian species have homologous primary structures (Einspanier et al., 1994; Reinert et al., 1996; Jonakova et al., 1998), their role in the fertilization process may be different. Proteins related to boar spermadhesins were found in bull and stallion seminal plasma (Einspanier et al., 1994; Calvete et al., 1995a). However, little attention has been paid to goat proteins homologous to boar spermadhesins.

The semen collected during the whole experimental period showed normal values concerning the parameters examined: volume, mass motility and progressive individual motility. These values were in accordance with those expected for male goats used in artificial insemination centers (Leboeuf et al., 2000).

Goat seminal plasma after ion-exchange chromatography on a DEAE-Sephacel column (Figure 1) yielded 6.47 ± 0.63 mg (mean ± SEM, N = 10) of a major retained fraction. The recovery of eluted proteins was 32.35 ± 3.16% (w/w) of starting material.

The RP-HPLC elution pattern of ion-exchange-bound proteins is shown in Figure 2. The protein studied was obtained in a pure state and the sequence of chromatography steps

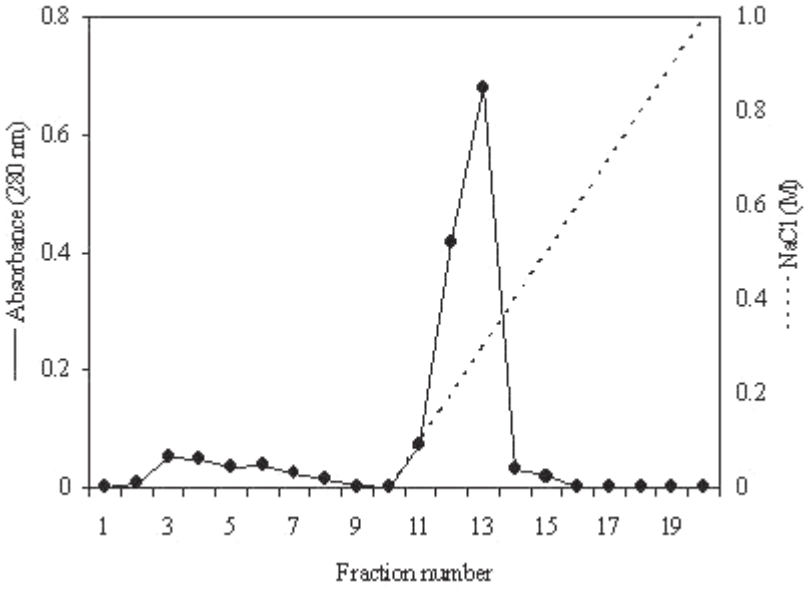

Figure 1. Ion-exchange chromatography of goat seminal plasma on a DEAE-Sephacel column. The column was washed with 0.02 M phosphate buffer, pH 7.0, at a flow rate of 30 mL/h and then eluted with a linear gradient of 0 to 1 M NaCl.

performed was efficient in purifying this protein as demonstrated by SDS-PAGE (Figure 2 - insert). This protein showed an apparent molecular mass of 12 kDa and was initially designated BSFP (buck seminal fluid protein) as previously described by Teixeira et al. (2002). The molecular mass was verified by the same authors using MALDI-TOF which exhibited a value of 12.591 kDa. The values for molecular mass are similar to that reported for AWN, a 14-kDa multifunctional protein, isolated from boar semen, which is one of the best structurally characterized spermadhesin so far (Ensslin et al., 1995; Jelinkova et al., 2003, 2004).

The N-terminal sequence of BSFP determined by automatic protein sequence analysis is presented in Figure 3A. BSFP exhibited N-terminal sequence homology to boar (AQN-1 and AWN), stallion (AWN) and bull (aSFP) spermadhesins (Figure 3B). In addition, BSFP also exhibited a high degree of similarity (50%) with the N-terminal sequence of boar AQN-1. Members of the spermadhesin family share 40 to 90% sequence identity but they are not functionally equivalent (Töpfer-Petersen et al., 1998).

Seminal plasma carbohydrate- and heparin-binding proteins of the spermadhesin family coat the surface of boar and stallion spermatozoa at ejaculation and are believed to participate in sperm capacitation and in the recognition between and initial protein-carbohydrate-binding mechanism of homologous gametes at fertilization (Töpfer-Petersen and Calvete, 1996; Reinert et al., 1996). However, each spermadhesin exhibits a different set of affinities depending on their glycosylated and aggregated state (Töpfer-Petersen, 1999). In addition, other spermadhesins do not show interaction with heparin and zona pellucida glycoconjugates, for example boar PSP-I (Varela et al., 1997), PSP-I/PSP-II complex (Solis et al., 1998) and bull aSFP (Romão et al., 1997). In our study, BSFP showed no heparin-binding capabilities as observed by heparin-

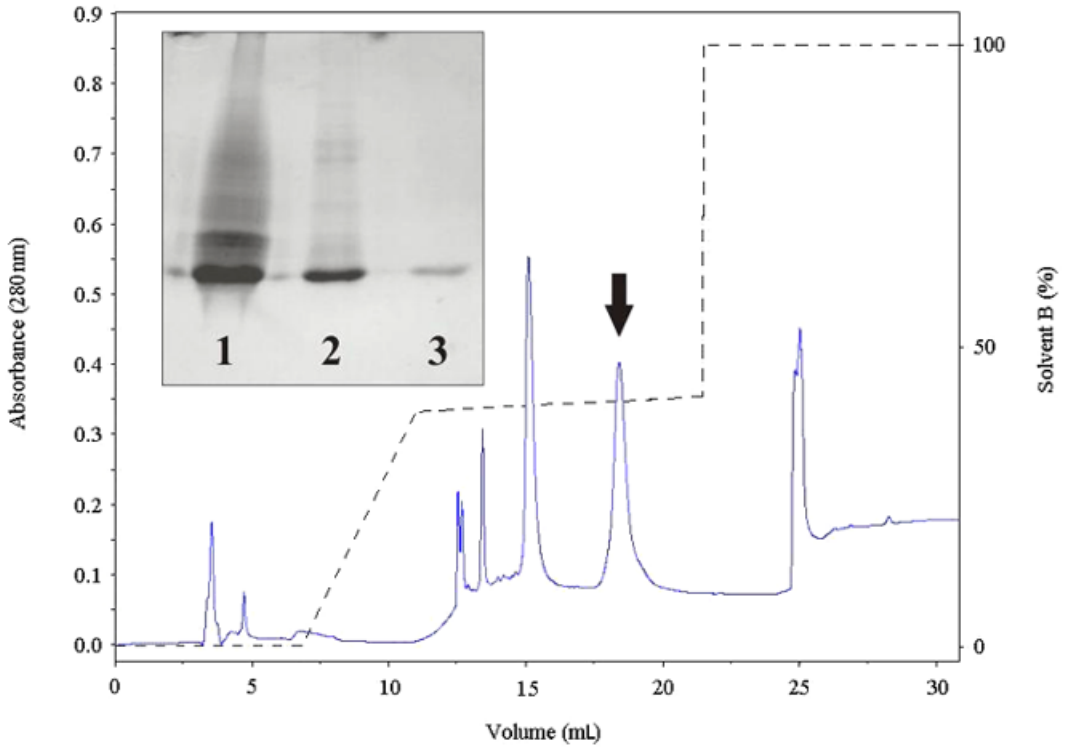

Figure 2. Reverse phase-high-performance liquid chromatography (RP-HPLC) pattern of DEAE-Sephacel-retained fraction. Proteins were eluted using 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). *Insert*, SDS-polyacrylamide gel electrophoretic analysis: *lane 1*, goat seminal plasma; *lane 2*, goat seminal plasma after DEAE chromatography, and *lane 3*, buck seminal fluid protein obtained from RP-HPLC (arrow in chromatogram).

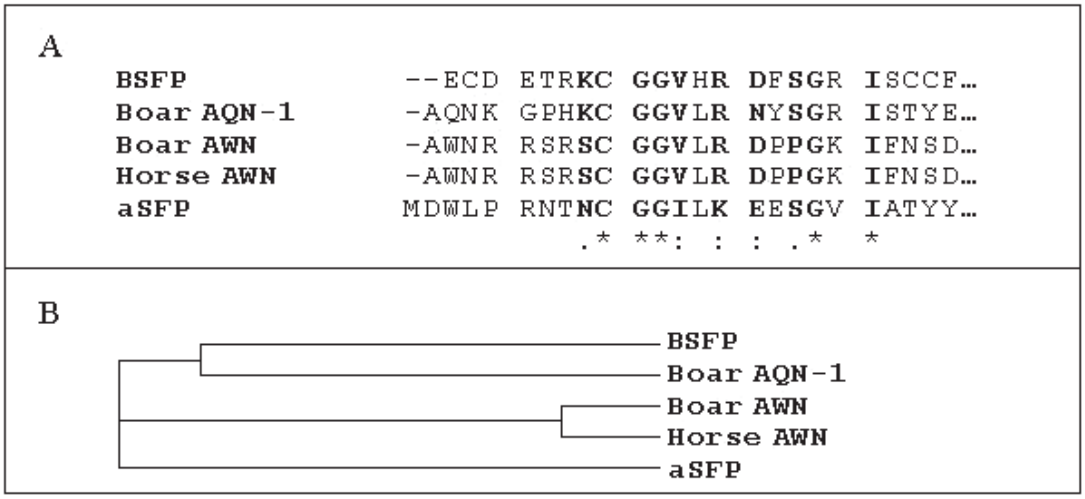

Figure 3. Comparison of N-terminal amino acid sequences of BSFP with boar (AQN-1, AWN), stallion (AWN) and bull (aSFP) spermadhesins. **A.** Alignment performed by CLUSTAL W. Asterisks mean identical residues in the column of all sequences; colons mean conserved substitutions, and periods mean semi-conserved substitution. **B.** Cladogram obtained from CLUSTAL W alignment with default settings. BSFP = buck seminal fluid protein.

Sepharose chromatography of the DEAE-Sephacel-retained fraction and SDS-polyacrylamide gel electrophoretic analysis (Figure 4).

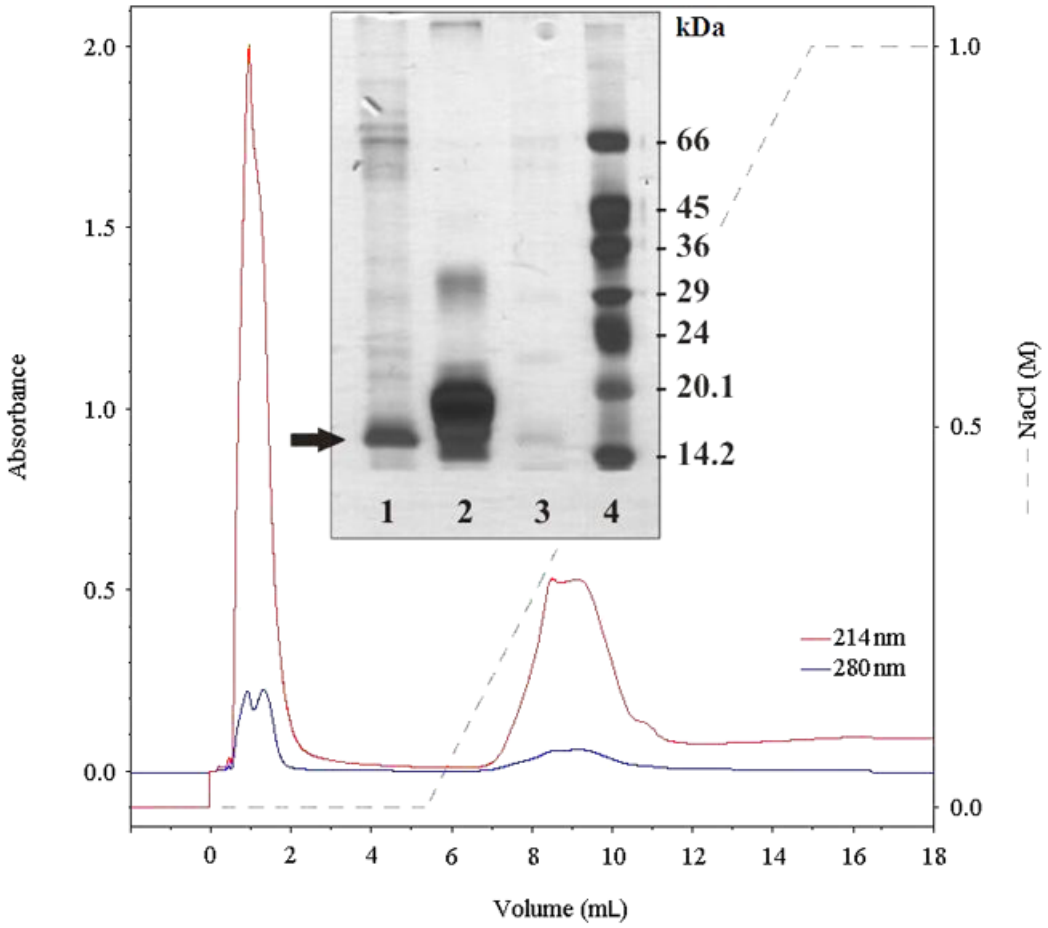

Figure 4. Heparin-Sepharose high-performance liquid chromatography pattern of DEAE-Sephacel-retained fraction. *Insert*, SDS-polyacrylamide gel electrophoretic analysis of protein fractions: *lane 1*, non-heparin-binding proteins; *lane 2*, boar spermadhesin (14 kDa); *lane 3*, heparin-binding proteins; *lane 4*, molecular mass markers: from top to bottom, bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

In goats, another group of proteins (GSP-14, GSP-15, GSP-20, and GSP-22 kDa) has been isolated from seminal plasma and separated according to heparin-affinity (Villemure et al., 2003). As with GSP-14 and GSP-15 kDa, BSFP also occurred in the non-heparin-binding fraction. However, based on their N-terminal amino acid sequence, BSFP and GSP are considered different proteins.

Spermadhesins from different domestic species, especially boar (Calvete et al., 1995b) and horse (Calvete et al., 1997), are obtained routinely by heparin-affinity chromatography as first step. Nevertheless, in the present study, the initial plasma fractionation by ion-exchange

chromatography was a simple and efficient approach in isolating BSFP. To our knowledge, this is a new approach to purifying a spermadhesin-family protein.

In conclusion, the present study indicates that goat seminal plasma contains a protein that is structurally related to proteins of the spermadhesin family. This protein can be efficiently isolated by ion-exchange chromatography. Further investigation of this protein would help enhance our understanding of capacitation and sperm-egg binding in goats.

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