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Methods for obtaining the enriched fraction of ram seminal vesicle proteins (RSVP14)

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ABSTRACT - The objective of the present study was to develop a methodology to obtain the enriched fraction of ram seminal vesicle protein 14 (RSVP14). The study was developed using Morada Nova rams, from which semen samples were collected weekly. Seminal plasma proteins were precipitated with cold ethanol, and then 6.15 mg/mL of total proteins were subjected to liquid gelatin affinity chromatography using a Gelatin-Sepharose matrix coupled to an automated chromatographic system. Proteins were eluted into four fractions (A, B, C, and D), in which A and B contained non-gelatin-binding proteins, and C and D fractions contained gelatin-binding proteins. Gels were analyzed by Quantity One software, in which five protein bands were detected in fraction D, with molecular weights between 12 and 30 kDa. The gelatin-binding proteins (fraction D) were loaded into a HiTrap™ Heparin HP affinity column. Two chromatographic fractions were separated (D1 and D2), in which D1 contained non-heparin-binding proteins, and D2 contained heparin-binding proteins. Proteins from the last two peaks were subjected to 12.5% SDS-PAGE and Western Blot. Two bands with molecular weight of 14 and 24 kDa, contained in fraction D1, were excised from gel and subjected to tandem mass spectrometry, identifying the proteins RSVP14 and RSVP24. Thus, the chromatographic methods of the present study are efficient to capture the enriched fraction of RSVP14.

Keywords: *Ovis aries*, proteomics, spermatozoa

1. Introduction

The seminal plasma is a complex physiological secretion originated from the testis, epididiymides, and accessory sex glands of the male reproductive tract, playing important roles in sperm capacitation (Plante et al., 2012) and other important events of male reproductive physiology, such as sperm motility and protection, acrosome reaction, fertilization, and initial embryonic development (Moura et al., 2018). The most abundant proteins in ruminant seminal plasma are members of two families of proteins, binder of sperm proteins (BSP) and spermadhesins. Proteins from BSP family bind to phospholipids and induce the efflux of cholesterol from the spermatic membrane to promote sperm capacitation (Plante et al., 2012).

Bergeron et al. (2005) indicated that BSP homologues identified in the seminal plasma of rams are defined as ram seminal vesicle proteins (RSVP). According to van Tilburg et al. (2013), the main proteins

present in ram seminal plasma are RSVP14 proteins (representing approximately 30% of the intensity of all spots identified in 2-D SDS PAGE). Amino acid sequences and disulfide bond assignments confirm the structural similarity between RSVP and BSP. Also, RSVP are specifically secreted by ram seminal vesicles (Fernández-Juan et al., 2006) and bind to the plasma membrane of sperm (Souza et al., 2012), inducing capactitation (Leahy et al., 2019).

Ovine BSP also act to protect sperm from damages caused by cryopreservation and detergent treatments (Luna et al., 2015; Barrios et al., 2005; Pini et al., 2018). The interaction of these proteins with the sperm membrane occurs due to the presence of fibronectin type II domains in RSVP of 14 kDa (Barrios et al., 2005), which is characteristic of BSP proteins. Besides, RSVP14 is part of the protein structure surrounding the sperm membrane, stabilizing its phospholipids (Barrios et al., 2005).

Therefore, given the importance of RSVP for sperm function and male fertility, the present study was conducted to develop a methodology to obtain an enriched fraction of RSVP14.

2. Material and Methods

The present study was accepted by the institutional animal ethics committee (case no. 1120270318).

Five 1-2-year old Morada Nova rams were used in the current study, and semen was collected for seven months weekly by means of electroejaculation, as reported before (Souza et al., 2010). Semen samples were mixed with a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) shortly after collection (Martins et al., 2013) and centrifuged (700 × *g*, 4 °C, 15 min) to separate the sperm cells from the supernatant. The supernatant was pipetted into clean tubes and centrifuged again (5000 × *g*, 4 °C, 60 min) to remove cell debris (Rodríguez-Villamil et al., 2016). Then, the samples from all animals were grouped in pools and stored at −80 °C until further analysis.

Ram seminal plasma proteins were precipitated according to Bergeron et al. (2005). In summary, nine volumes of pure ethanol stored at low temperature were added to 15 mL of seminal plasma in agitation for 90 min at 4 °C and then centrifuged at 10,000 × *g* for 10 min. After three subsequent washes with ethanol, the precipitates were solubilized in 50 mM ammonium bicarbonate and lyophilized. About 370 mg of dry seminal plasma powder were recovered after this protocol. Lyophilized seminal proteins were resuspended in 30 mL of binding buffer (Solution A: 40 mM Tris, 2 mM $CaCl_2$, pH 7.4), purchased from GE Healthcare (Piscataway, NJ, USA) and packed into 15 microtubes of 2000 µL and stored at −80 °C. An aliquot of this pool was used to determine the soluble protein concentration (Bradford, 1976).

The soluble protein concentration of seminal plasma pool was 12.3 mg/mL, and 6.15 mg/mL were applied for chromatographic runs (the retention capacity of the gelatin column was 4.5-8.0 mg). Fractions containing 6.15 mg/mL of precipitated seminal plasma proteins were subjected to gelatin affinity chromatography using a 25-mL-Gelatin Sepharose 4B matrix packed in an empty XK 16/20 column (GE Healthcare; Piscataway, NJ, USA). The column was equilibrated with binding buffer (Solution A: 40 mM Tris, 2 mM $CaCl_2$, pH 7.4), coupled to the Äkta Prime Plus chromatography system (GE Healthcare, Piscataway, NJ, USA). A low initial flow of 1 mL/min binding buffer was applied to allow the gelatin-binding proteins to interact with the column. After 5 min, the flow rate was increased to 2 mL/min for 40 min, and gelatin binding proteins were eluted using 8 M of urea added to the binding buffer. Peaks containing non-gelatin-binding and gelatin-binding proteins were determined after this chromatography run. Fractions with gelatin-binding components were concentrated using 10-kDa filters (VivaSpin MWCO 10 kDa, GE Healthcare, Piscataway, NJ, USA) and the material retained by the filters was quantified according to Bradford's method (Bradford, 1976). Fractions without affinity to gelatin were treated in a similar manner. To estimate the percentage of proteins bound to gelatin, peak integration was performed using PrimeView Evaluation Software (GE Healthcare, Piscataway, NJ, USA) (Rodríguez-Villamil et al., 2016). Gelatin-binding proteins obtained at this first chromatographic run were stored at −20 °C for the next step.

Concentration of soluble gelatin-binding proteins was determined by Bradford's method, and 1.72 mg/mL of gelatin-binding proteins was applied in the next chromatographic run. Briefly, proteins were diluted in 100 mL of binding buffer (Sol. A: 40 mM Tris, 2 mM $CaCl_2$, pH 7.4), respecting the maximum retention capacity of the heparin column (3 mg). The fraction of proteins with affinity to gelatin was separated by heparin affinity chromatography using a HiTrap Heparin HP column of 1 mL (GE Healthcare, Piscataway, NJ, USA), coupled to the Äkta Prime Plus chromatography system (GE Healthcare, Piscataway, NJ, USA). The column was equilibrated with binding buffer. An initial flow of 0.5 mL/min of binding buffer was applied to allow heparin-binding proteins to interact with the column. After 5 min, the flow was increased to 1 mL/min and for 30 min and the retained proteins were eluted using 1 M of sodium chloride (NaCl) added to the binding buffer. After this second chromatographic run, two peaks were formed, corresponding to non-heparin-binding and heparin-binding proteins. These two peaks were separated and concentrated using 10-kDa filters. The concentration of soluble proteins retained in the filter was also determined (Bradford, 1976). To estimate the percentage of non-heparin-binding and heparin-binding proteins after the chromatographic run, peak integration was performed and analyzed using PrimeView Evaluation Software, with the baseline adjusted to 0 mAu at 280 nm. The concentrations of heparin-binding and non-heparin binding proteins were approximately 1.2 and 1.0 mg/mL, respectively. The two fractions were stored at −20 °C for further analysis.

For 1-D SDS-PAGE, a volume containing 20 µg of proteins from each fraction obtained from affinity chromatographic runs were mixed with 20 µL of sample buffer [0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.2 M Dithiothreitol (DTT), 0.02% bromophenol blue; GE Healthcare, Piscataway, NJ, USA], boiled for 90 s, and pipetted to the wells of a stacking gel (4% of acrylamide), placed on a 12.5% gradient polyacrylamide gel. An equal amount of seminal plasma proteins was also applied as a positive control. In one well of the stacking gel, 10 µL of Amersham ECL Full-Range Rainbow Molecular Weight (GE Healthcare, Piscataway, NJ, USA) were loaded to allow molecular weight estimation of the protein bands. The 1-D SDS-PAGE was run in a SE600 Ruby apparatus (GE Healthcare, Piscataway, NJ, USA), at 500 V, 25 mA/gel, 90 W. The gel was stained with Coomassie Brilliant Blue (CBB-R250) for 12 h and destained after several washes in a solution containing methanol (40%) and acetic acid (10%) in double-distilled water. After destaining, the gel was scanned at 300 dpi (Image Scanner, GE Healthcare, Piscataway, NJ, USA) and saved as a TIFF file. The image was analyzed using Quantity One software, v.4.6.3 (Bio-Rad, Rockville, MD, USA). The molecular weight of each band was determined according to the molecular marker using a point-to-point regression model available in the Quantity One software.

Proteins separated by 1-D SDS-PAGE were destained and trypsin-digested as previously described in detail (Martins et al., 2013; Moura et al., 2007). In summary, the selected bands were cut into approximately 1 mm^3 pieces, transferred to clean tubes and washed three times with solution containing acetonitrile (50%) and ammonium bicarbonate (50%; 25 mM at pH 8.0) and washed again twice with 200 µL acetonitrile for 5 min and dried under vacuum. The bands were incubated with trypsin for 20 h at 37 °C. The peptides were then extracted by triple washing with 5% trifluoroacetic acid in 50% acetronitrile and ammonium bicarbonate (50 mM) for 30 min. Supernatants were concentrated in microtubes and vacuum-dried (Eppendorf, Hauppauge, NY, USA). The peptides were separated on a C18 BEH300 column (100 µm × 100 mm) using the nanoAcquity ™ system (Waters Corp, Milford, CT, EUA) and eluted at 600 µL/min with acetronitrile gradient (5–85%) containing 0.1% of formic acid. The liquid chromatography system was connected to a nanospray mass ionization source (Synapt hdms system, Waters Corp, Milford, CT, EUA). The mass spectrometer was operated in positive mode using 90 °C capillary and 3.5 kV voltage. Instrument calibration was performed using double protonated [Glu1] -fibrinopeptide B fragments (m/z 785.84), and the Lock-mass used during acquisition was the intact ion. The LC-MS/MS procedure was performed according to the data-dependent acquisition (DDA) method by selecting double or triple charge precursor ions MS/MS. The ions were fragmented by induced collision dissociation using argon as the collision gas and collision energy ramp that varied according to the charge state of the selected precursor ion. Data collection was performed at a range of m/z 300–2100 for MS sampling (1 scan/s), identifying ions with m/z ranging from 50–2500 for MS/MS. Data were collected using MassLynx 4.1 software,

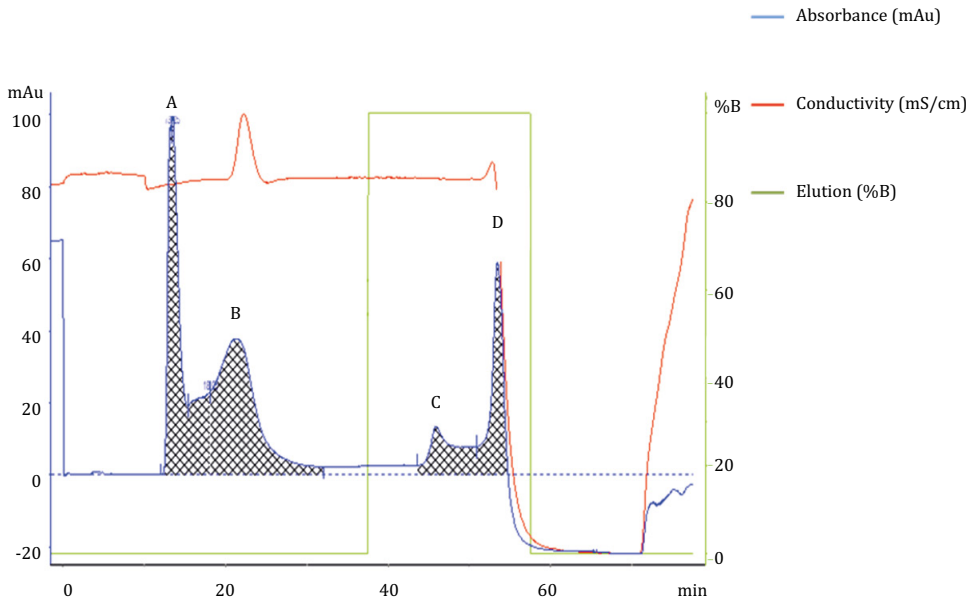
processed using Protein Lynx Global Server 2.4 server (Waters Corp, Milford, CT, EUA), and converted to peak list text file (pkl) for database searching.

The ionic spectra obtained for each pkl peptide were searched using the MASCOT search tool (Matrix Science, London, UK, v.2.6) in the NCBInr database using the MS/MS ion search mode. For the search, we considered loss values of at most one tryptic cleavage, monoisotopic peptides with a load of +1, +2, and +3, with variable methionine residue oxidation and fixed variation of carbamidomethylated cysteine residues. Identification was considered unambiguous when the protein score was significant ($P<0.05$), and due to the close coincidence of the theoretical and experimental protein molecular weight and isoelectric point.

Twenty micrograms of seminal plasma and 20 µg of non-gelatin-binding and gelating-binding were subjected to molecular separation by 12.5% SDS-PAGE and then transferred (at 45 mA for 90 min) to a PVDF Hybond-P membrane (GE Lifesciences, Piscataway, NJ, USA) using a TE 70 transfer unit (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked overnight and incubated with primary antibody as described previously by Souza et al. (2012), at 4 °C with 30 mL of PBS with 0.5% Tween-20 (PBS-T, GE Healthcare, Piscataway, NJ, USA) containing skimmed milk (5% w/p), under agitation, followed by 1 h incubation with antibody against the sperm protein ligand (1:6000 for anti-BSP) based on the protocol described by Moura et al. (2007), with modifications. The PVDF membranes were then washed three times in 1X Phosphate-Buffered Saline and Tween Detergent (PBS-T, GE Healthcare, Piscataway, NJ, USA), and incubated for 1 h with anti-rabbit IgG (1:5000, Abcam, UK) along with 30 mL PBS with 0.5% Tween-20 (PBS-T, washed again three times in PBS-T and washed once with Tris-HCl 50 mM). Immunoreaction was visualized by exposing the membranes to BCIP/NBT alkaline phosphatase substrate, pH 9.5 (Thermo Scientific, Waltham, MA, EUA). The reaction was stopped by washing the membranes with ultrapure water.

3. Results

Based on chromatographic profiles, gelatin-binding proteins represented approximately 30% of ram seminal plasma total protein. Chromatograms of gelatin affinity chromatography presented four peaks well separated and eluted in 13, 20, 45, and 53 min, respectively (Figure 1). Fractions A and B

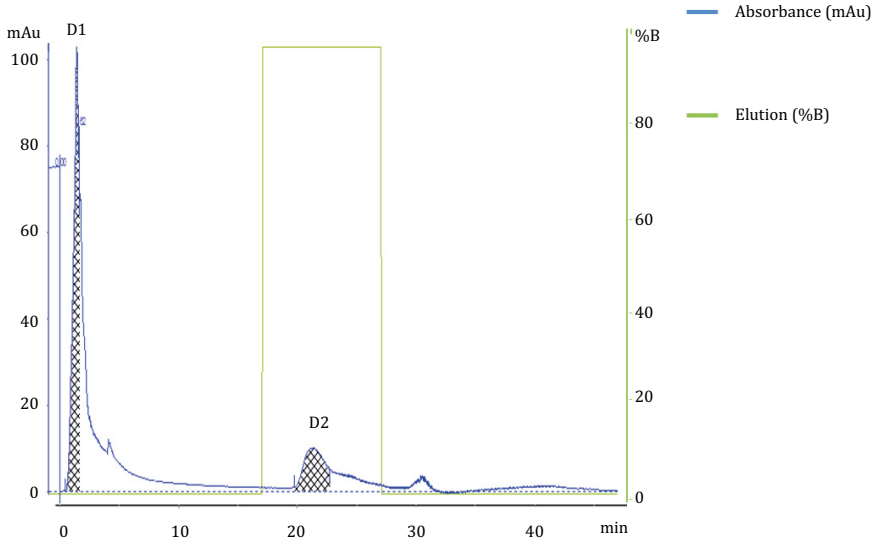


A: fraction A - non-gelatin-binding proteins; B: fraction B - non-gelatin-binding proteins; C: fraction C - gelatin-binding proteins; D: fraction D - gelatin-binding proteins with the highest concentration of RSVP14.

Figure 1 - Gelatin affinity chromatography with ovine seminal plasma proteins.

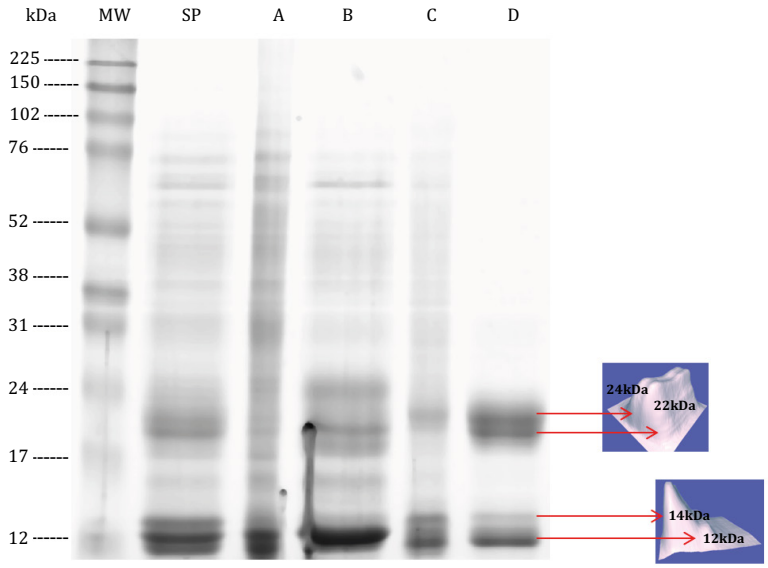
were eluted during the column washing with binding buffer, for 30 min, with absorbances of 100 and 40 mAu, respectively. The last two peaks, grouped in fractions C and D, were eluted in the presence of 8 M urea for 60 min, with an absorbance of 18 and 60 mAu, respectively (Figure 1). After separation by heparin affinity chromatography, the non-heparin-binding proteins represented the peak of interest with 30.64% of proteins obtained in the first chromatographic step. Chromatograms of heparin affinity chromatographies presented two peaks eluted in 3 and 20 min, respectively (Figure 2).

Gelatin-binding proteins and whole seminal plasma proteins separated by 1-D SDS PAGE were analyzed and compared using Quantity One software. Bands representing fractions A, B, C, and D indicated the presence of 28 proteins with molecular weights between 14 and 102 kDa (Figure 3). Fraction D



D1: fraction D1 - non-heparin-binding proteins; D2: fraction D2 - heparin-binding proteins.

Figure 2 - Heparin affinity chromatography of gelatin binding proteins obtained in the previous chromatographic step.

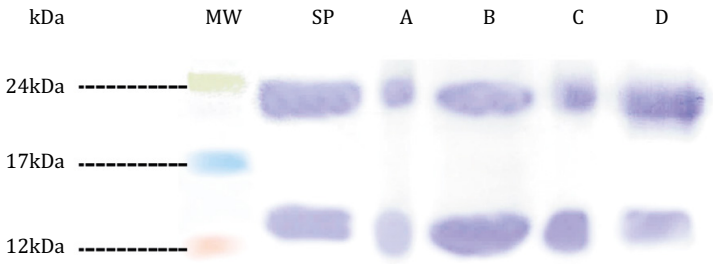


Images indicated by red arrows are 3D representations of protein bands generated by Quantity One software (Bio-Rad, Rockville, MD, USA), showing four protein bands with molecular weights between 12 and 24 kDa. Whole seminal plasma refers to seminal plasma samples prior to any chromatographic analysis.

Figure 3 - SDS-PAGE of whole seminal plasma proteins (SP) and gelatin-binding proteins without (A and B) and with (C and D) affinity to gelatin.

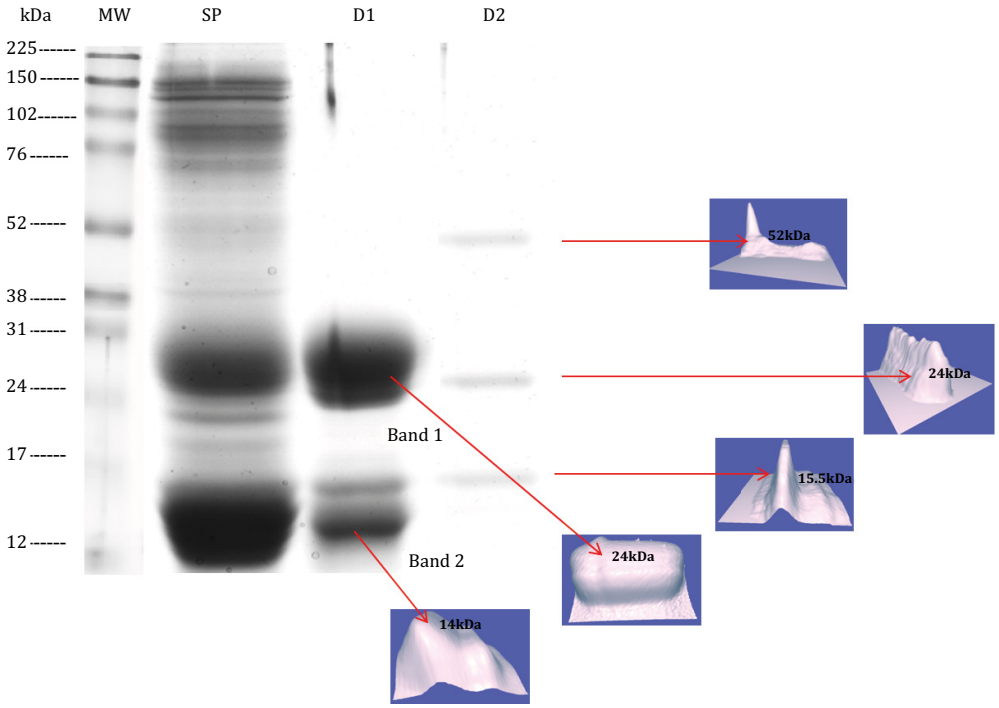
(Figure 3) represented the peak of interest, with 28.44% of proteins bound to gelatin. Fractions A and B (Figure 3) included non-gelatin-binding proteins with 12-73 kDa. An immunodetection test was performed with anti-BSP antibodies (Figure 4), and the four peaks of the gelatin column reacted to that antibody. The chromatographic conditions applied to obtain fraction D were efficient to separate the proteins into two peaks, grouped in D1 (non-heparin-binding proteins) and D2 (heparin-binding proteins) (Figure 5).

One-dimensional electrophoresis indicated that the enriched fraction shown in lane D1 had only two bands with molecular weights of 14 and 24 kDa. As determined by mass spectrometry, band 1 contained binder of sperm 1 precursor (*Ovis aries*), and band 2 contained binder of sperm 5 precursor (*Ovis aries*; Table 1). Western blots using anti-BSP antibodies confirmed the presence of BSP in heparin-binding (D1) and non-heparin-binding (D2) fractions (Figure 6).



MW - molecular weight; A - fraction A = non-gelatin-binding proteins; B - fraction B = non-gelatin-binding proteins; C - fraction C = gelatin-binding proteins; D - fraction D = gelatin-binding proteins with the highest concentration of RSVP14.

Figure 4 - Western blotting performed using anti-binder of sperm antibodies.



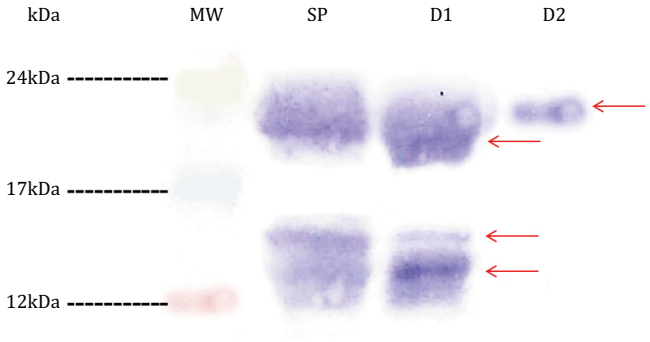
Gelatin-binding proteins loaded into heparin chromatographic columns were those shown in fraction D of Figure 3. Bands 1 and 2 were excised from gels, digested with trypsin, and subjected to mass spectrometry.
Images indicated by red arrows are 3D representations of protein bands generated by Quantity One software (Bio-Rad, Rockville, MD, USA).
Whole seminal plasma refers to seminal plasma samples prior to any chromatographic analysis.

Figure 5 - SDS-PAGE of whole seminal plasma proteins (SP) and heparin-binding proteins without (D1) and with (D2) affinity to heparin.

Table 1 - Binder of sperm proteins (BSP) precursors enriched fractions from *Ovis aries* seminal plasma identified by one-dimensional SDS-PAGE and tandem mass spetrometry

Protein and bands	Protein name	NCBI acession number	MS/MS protein score	Sequence covered (%)	Matched peptide	Ion score	m/z	z
Band 1	Binder of sperm 1 precursor (*Ovis aries*)	NP_001137137.1	83	17	(55) HFDCTFHGSIFPWCSLDADYVGR (77) (55) HFDCTFHGSIFPWCSLDADYVGR (77)	83 69	697.5483 929.7413	4 3
Band 2	Binder of sperm 5 precursor (*Ovis aries*)	NP_001087251.1	90	13	(122) CTTEGSAFGLAWCSLTEYFER (142)	90	1243.0447	2

Band numbers refer to those shown in Figure 5.



MW - molecular weight; SP - seminal plasma (control); D1 - fraction D1 (non-heparin-binding proteins); D2 - fraction D2 (heparin-binding proteins). Red arrows point to a specific marking for binder of sperm proteins in the two fractions.

Figure 6 - Western blotting performed using anti-binder of sperm antibodies with proteins bound and non-bound to heparin.

4. Discussion

In the present study, we described a new method to obtain a RSVP14 enriched fraction from ram seminal plasma, consisting of two sequential steps of affinity chromatography. Binder of sperm proteins have affinity to gelatin, heparin, and other glycosaminoglicans as the result of hydrophobic and ionic interactions (Plante et al., 2016; Gasset et al., 1997). Also, BSP proteins have conserved structures composed of a N-terminal domain variable and two fibronectin type II domains arranged in tandem (Plante et al., 2016). Our method, thus, used BSP chemical attributes to obtain an RSVP14 enriched fraction of complex seminal plasma samples from rams.

In the present study, the group of proteins with 14-15 and 22-24 kDa were the most predominant in fraction D (Figure 3). According to Bergeron et al. (2005), such proteins are defined as RSVP-14, RSVP-15, RSVP-22, and RSVP-24 kDa. We found results (Figure 3) similar to those described by Villemure et al. (2003), who suggested that proteins present in fractions A and B could be BSP homologues along with other proteins of the same molecular weight.

Bovine BSP1 is glycosylated, being visually differentiated with two protein isoforms by immunoblot analysis. The BSP proteins in bulls, stallions, wild boars, and humans tend to form clusters in solution (Calvete et al., 1995; Kumar et al., 2008; Manjunath et al., 1988; Plante et al., 2016). In the study of Villemure et al. (2003), the comparison of the N-terminal sequence of proteins from RSVP family indicated a high degree of structural relationships between RSVP. This suggests the existence of different glycoforms of RSVP14 in ovine seminal plasma, which proves that RSVP proteins exist in different molecular forms and glycoforms. Martins et al. (2013) reported that RSVP of 14 and 22 kDa had no affinity to heparin, results that are in agreement with the Western blots conducted in our study.

The BSP homologues of other species such as goats (Villemure et al., 2003) and bisons (Boisvert et al., 2004) do not bind to heparin either. Amino acid substitutions can modify the affinity of molecules to heparin (Ward, 2010), so it is possible that differences in amino acids and sequences of fibronectin II domains in RSVP prevent them from binding to heparin (Martins et al., 2013).

The RSVP contained in D1 fraction (Figure 5) did not bind to heparin, similarly to the result described by Villemure et al. (2003) in goats. Binder of sperm proteins play roles in sperm capacitation, sperm interaction with the oviduct epithelium, and fertilization. Also, BSP interact with components of semen extenders, suggesting that BSP can be used for the development of new protocols for sperm cryopreservation and artificial insemination (Moura et al., 2018).

Artificial insemination in sheep is challenging but a feasible technique. Early studies have observed a wide variety of sublethal freezing effects of cryopreservation on sperm (Peris et al., 2007). Thus, in the study developed by Ari and Daskin (2010), the addition of ram seminal plasma protected and significantly improved the stabilization of acrosome membrane of goat sperm post-thaw. In the study by Barrios et al. (2005), seminal plasma proteins of approximately 14 and 20 kDa were responsible for protection of ram sperm membrane. More recently, studies conducted in Australia indicated that seminal plasma proteins, including BSP, can prevent damages to ram sperm caused by cryopreservation (Barrios et al., 2005; Luna et al., 2015; Pini et al., 2018). Thus, there seems to be sufficient pieces of evidence to support the concept that certain seminal plasma proteins are beneficial to sperm and protect these cells during cryopreservation. This knowledge can be exploited to improve methods of commercial sperm freezing (Pini et al., 2018).

5. Conclusions

Affinity chromatographic methods used in the present study were efficient to obtain an enriched fraction of RSVP14 from ram seminal plasma. This is a significant step to obtain RSVP from whole seminal fluid, but the use of additional chromatographic approaches, such as ionic exchange or gel filtration, are still required to obtain highly purified RSVP. Enriched RSVP14 fractions or purified RSVP can be used in studies designed to understand the precise roles of the major seminal plasma proteins of rams. Purified seminal proteins can also be used in extenders to improve the results of commercial freezing and the outcome of artificial insemination in ovine species.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: B.A. Felix, J.A.M. Martins, F.C.P. Santos and A.A. Moura. Data curation: B.A. Felix. Formal analysis: B.A. Felix, K.S. Otávio, J.A.M. Martins, A.L.M.C.S. Velho and F.R. Vasconcelos. Funding acquisition: A.A. Moura. Investigation: B.A. Felix, K.S. Otávio, J.A.M. Martins, F.C.P. Santos, F.R. Vasconcelos and A.A. Moura. Methodology: B.A. Felix, K.S. Otávio, J.A.M. Martins, F.C.P. Santos and A.L.M.C.S. Velho. Project administration: B.A. Felix and A.A. Moura. Resources: A.A. Moura. Supervision: B.A. Felix, J.A.M. Martins, F.R. Vasconcelos and A.A. Moura. Validation: B.A. Felix, J.A.M. Martins and A.A. Moura. Visualization: B.A. Felix, K.S. Otávio, J.A.M. Martins and A.A. Moura. Writing-original draft: B.A. Felix and K.S. Otávio. Writing-review & editing: B.A. Felix, K.S. Otávio, M.J.B. Bezerra and A.A. Moura.

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