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Differential abundances of four forms of Binder of SPerm 1 in the seminal plasma of *Bos taurus indicus* bulls with different patterns of semen freezability

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ABSTRACT

The Binder of SPerm 1 (BSP1) protein is involved in the fertilization and semen cryopreservation processes and is described to be both beneficial and detrimental to sperm. Previously, the relationship of BSP1 with freezability events has not been completely understood. The objective of this work was to determine the differential abundance of the forms of the BSP1 protein in cryopreserved seminal plasma of *Bos taurus indicus* bulls with different patterns of semen freezability using proteomics. A wide cohort of adult bulls with high genetic value from an artificial insemination center was used as donors of high quality, fresh semen. Nine bulls presenting different patterns of semen freezability were selected. Two-dimensional gel electrophoresis showed differential abundance in a group of seven protein spots in the frozen/thawed seminal plasma from the bulls, ranging from 15 to 17 kDa, with pI values from 4.6 to 5.8. Four of these spots were confirmed to be BSP1 using mass spectrometry, proteomics, biochemical, and computational analysis (Tukey's test at $P < 0.05$). The protein spot weighing 15.52 ± 0.53 kDa with a pI value of 5.78 ± 0.12 is highlighted by its high abundance in bulls with low semen freezability and its absence in bulls presenting high semen freezability. This is the first report showing that more than two forms of BSP1 are found in the seminal plasma of Nelore adult bulls and not all animals have a similar abundance of each BSP1 form. Different BSP1 forms may be involved in different events of fertilization and the cryopreservation process.

1. Introduction

Selection of bulls with high genetic value is an essential tool to improve farmers' herds in regard to the production of beef cattle [1]. At artificial insemination (AI) centers, it is necessary to assess a variety of breeding bulls to accurately select animals that possess high quality and sufficient freezability of the semen [2]. Bulls presenting high reproductive capacity in natura can produce semen sensitive to cryopreservation [3]. Routine assessments of semen [4] and systems for computer-assisted sperm analysis [5], which are widely used by AI centers, do not accurately determine the potential freezability of semen.

After cryopreservation of bull semen, the spermatic viability may be reduced by up to 50%, but cryopreservation is a poorly understood process at the molecular level [3,6].

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The presence of Binder of SPerm (BSP) proteins [7] is known to influence the success of cryopreservation [8], along with other biomolecules present in the complex mixture of the seminal plasma [9]. BSP proteins belong to a family of heparin-binding proteins and represent approximately 70% of the total protein content of the plasma [10]. They are involved in different steps of bovine fertilization, including sperm capacitation. BSPs promote a cholesterol efflux from sperm membranes, which increases their fluidity and modulates membrane stability [11]. During cryopreservation, the cholesterol efflux can lead to a decrease in sperm resistance to cold [3].

One of the most abundant BSP proteins, BSP1, is described to be a mixture of the BSP-A1 and BSP-A2 proteins [9] and is multifunctional in the seminal plasma [3,12]. In an attempt to unravel the physiological events involved in sperm fertility and the cryopreservation of the sperm of bulls, BSP1 has been widely studied [3,12–14]. BSP1 plays a role in forming an oviductal sperm reservoir by enabling the sperm to bind to the oviductal epithelium, maintaining bull sperm motility. In addition, BSP1 participates in the changes in the sperm plasma membrane by stimulating the cholesterol efflux, causing cryoinjuries to the frozen semen that arise from premature semen capacitation and acrosome reaction [3,11,12]. Therefore, BSP1, alongside other BSP proteins, may be both beneficial and detrimental to sperm [8], supporting the idea that this singular protein has multiple functions.

Despite the many studies concerning cryoinjuries in semen, information about the presence of different forms of BSP1 and the probable involvement of these forms in different events in reproduction and cryopreservation is scarce. Two-dimensional electrophoresis (2-DE) has proven to be helpful in the study of glycoproteins that have small differences in mass and charge [15]. The objective of this work was to use proteomics to determine the differential abundance of the forms of the BSP1 protein in cryopreserved seminal plasma of *Bos taurus indicus* bulls with different patterns of semen freezability.

2. Materials and methods

2.1. Materials

All materials were of the highest grade available. Chemicals and reagents were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA) or from Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. Specific materials for electrophoresis procedures were purchased from GE Healthcare (Life Sciences, USA).

2.2. Animals, sample collection, and semen analysis

Nine healthy fertile *Bos taurus indicus* adult bulls with an average age of 7 years (ranging from 3 to 10) were selected from a subset of a wide cohort of recurrent donors of semen from an AI center in the city of Magda, São Paulo, Brazil (20°38′38″ S, 50°13′34″ W, 526 m). All bulls were kept under similar handling and feeding conditions and were confirmed to have low-freezability semen for more than a year. Semen collection was performed using an artificial vagina. Semen samples were assessed as routinely performed at the AI center.

In this work, the physical and morphologic evaluations of the fresh (F) semen were performed immediately after collection while maintained at 37 °C, as recommended by the Brazilian Animal Reproduction College [4]. For semen cryopreservation, the sperm concentration was determined and adjusted to 25 million sperm per dose by the addition of a cryoprotector on the basis of citrate, egg yolk, and glycerol [16]. Semen samples were maintained at 4 °C following preservation inside of thin 250-μL cryovials in liquid nitrogen, as regularly performed at the AI center. Frozen samples were thawed in an ice-bath, and the physical evaluation of the cryopreserved semen was performed. Bull selection focused on animals that produced ejaculates of high sperm quality for F semen but did not present similar values for straight progressive sperm motility in the same semen after a frozen/thawing (FT) process. The accepted parameter values in F semen included greater than 70% straight progressive sperm motility, greater than 3 (scale from 0 to 5) sperm vigor, less than 15% major sperm defects, less than 15% minor sperm defects, and less than 30% of sperm abnormalities for total sperm defects. For FT semen, the minimum accepted value for straight progressive sperm motility was 30%, while the other parameter values were maintained.

The straight progressive sperm motility and the sperm vigor were assessed by placing a 10-μL aliquot of semen between a slide and a coverslip preheated to 37 °C and analyzing the sample under an optical microscope at a magnification of 200 to 400 times [4]. The spermatozoa defects were evaluated on a wet preparation. An aliquot of semen diluted in buffered formol-saline was used for analysis by phase-contrast microscopy at a magnification of 1000 times under a drop of immersion oil [17]. For each ejaculate, a set of 400 cells were examined, and the sperm defects were measured as a percentage according to the classification criteria adopted by Blomm [18], as recommended by the CBRA [4]. All analyses were performed in at least three technical replicates.

2.3. Seminal plasma preparation

Immediately after collection, a 6.0-mL aliquot of F semen from each bull was centrifuged at 150 × *g* for 10 minutes to remove the spermatozoa and again centrifuged at 700 × *g* for 10 minutes to remove cellular debris. Each recovered supernatant was filtered at 0.22 μm to obtain seminal plasma, followed by cryopreservation inside of thin 250-μL cryovials in liquid nitrogen. The 24 frozen cryovials were transferred to an ultra-freezer at −80 °C.

2.4. Determination of soluble protein concentration

Quantification of soluble proteins in FT seminal plasma samples was performed by three procedures as follows: (1) the Coomassie Brilliant Blue (CBB) method [19], (2) the CBB method for samples precipitated with pure acetone, and (3) the bicinchoninic acid (BCA) method [20]. Three FT samples from each bull were singly thawed and re-centrifuged (10,000 × *g* for 60 minutes at 4 °C). Then, the samples

were evaluated by the CBB and BCA methods. Additionally, the same samples were singly precipitated using three volumes of pure cold acetone for 3 hours at −20 °C and centrifuged at 20,100 × g for 30 minutes at 4 °C. The precipitated proteins were re-solubilized in 50 μL of a sample solution containing 7 mol L^{-1} urea, 2% wt/vol CHAPS (3-3-cholamidopropyl dimethylammonio-1-propanesulfonate), and 2 mol L^{-1} thiourea, following quantification by the CBB method. Bovine serum albumin was used to create analytical calibration curves for all methods. The protein assays were performed in three technical replicates for each semen sample.

2.5. One-dimensional gel electrophoresis on Tricine (SDS-Tricine-PAGE)

SDS-Tricine-PAGE [21] was performed running a three stage gel (separation: 14.0% T, 3.0% C; intermediate: 9.9% T, 3.0% C; stacking: 5.1% T, 2.6% C) at 100 V for 15 minutes, 80 V for 20 minutes, and then 60 V for a total of 6.5 hours [22]. A mini-gel apparatus (Mini-PROTEAN Tetra Cell; Bio-Rad) was used. Samples contained 50 μg of soluble proteins. Broad-range molecular weight (MW) markers (Bio-Rad) allowed comparison among samples. The gels were stained with Coomassie blue [23].

2.6. Two-dimensional gel electrophoresis and analysis of gel images

Two-dimensional gel electrophoresis (2-DE) was performed [24] under two conditions. First, we used a pH 3 to 10 immobilized pH gradient (IPG) strips (7-cm, linear) for seminal plasma samples from the nine selected bulls. Later, we used pH 4 to 7 IPG strips (7-cm, linear) for samples from two bulls to exploit a specific group of proteins. For 2-DE using the pH 3 to 10 IPG strips, three cryovials containing FT seminal plasma from each bull were used, and one gel was developed using the content of each cryovial, for a total of 27 gels. Each sample containing 120 μg of soluble protein precipitated with acetone was solubilized in rehydration buffer (containing 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 40-mM dithiothreitol (DTT), 0.5% vol/vol free ampholytes (IPG buffer pH 3–10), 2% wt/vol CHAPS, and 0.001% wt/vol Bromophenol blue). The sample was brought to 125 μL using the DeStreak rehydration solution (GE Healthcare). Each sample was then loaded in a reswelling tray, overlaid with the IPG strip, and allowed to re-hydrate for 20 hours. For 2-DE using the pH 4 to 7 IPG strips, similar conditions were used, except the IPG strips and the free ampholyte (IPG buffer) were specific for pH 4 to 7.

Isoelectric focusing was carried out using an Ettan IPGphor3 apparatus (GE Healthcare) according to the manufacturer's instructions. After focusing, the IPG strips were incubated for 15 minutes in reducing buffer (6 mol L^{-1} urea, 30% vol/vol glycerol, 2% wt/vol SDS, 1% wt/vol DTT, and 75 mmol-L^{-1} Tris-HCl, pH 8.8) and re-equilibrated for an additional 15 minutes in alkylation buffer (similar to reducing buffer, containing 2.5% wt/vol iodoacetamide instead of DTT). The equilibrated IPG strips were then loaded and fixed with 5% wt/vol agarose (in SDS-PAGE running buffer) on the top of homogeneous 14% SDS-PAGE gels [21]. A mini-gel apparatus was used, and it was run at 125 V for approximately 180 minutes. The proteins in the gel were visualized using Coomassie blue G-250 staining [23].

The 2-DE gels were scanned at 300 dpi using an Image Scanner III (GE Healthcare). The files saved as .mel format were analyzed by Image Master III (GE Healthcare). For gel analysis, a unique master gel representative of the sample was obtained using a set of three gels corresponding to the technical replicates. Proteins in key regions of the master gel were chosen as landmarks. Matching of spots was achieved after several rounds of comparisons by automatic detection of the spots in each representative gel. Final spot matches were achieved by checking each spot in each gel with the respective pattern of the master. The spot volumes were normalized to the total spot volume of the gel. The percentage volume of each spot was used to estimate the differential abundance of the protein among the samples.

2.7. In-gel protein digestion

Each protein spot of interest was excised individually from the three gels for each sample type. The spots were destained with three washes of 400 μL of 50% vol/vol acetonitrile (ACN) containing 25 mmol L^{-1} ammonium bicarbonate. Gel pieces were then dehydrated by two incubations with 200 μL of absolute ACN for 5 minutes using a vacuum concentration system (Savant SpeedVac; Thermo Scientific, USA). Dry gel pieces were incubated for 20 hours at 37 °C with trypsin (166 ηg.$spot^{-1}$; Promega, Sequencing Grade Modified Trypsin, cat #V5111, USA) for in-gel trypsin digestion [25]. The peptides were extracted from gel pieces by incubating three times with 50 μL of trifluoroacetic acid (TFA) and 50% vol/vol ACN in ammonium bicarbonate (50 mmol L^{-1}), for 30 minutes. The supernatants were concentrated to a final volume of 10 μL using a vacuum concentration system. Blank gel pieces were used as negative controls. Disposable C18 resin ZipTip pipette tips (Millipore, USA) cleaned up the samples according to the manufacturer's recommendations. The samples were eluted with 80% vol/vol ACN and 0.1% vol/vol TFA into a new-tube and concentrated to 10 μL for protein identification by mass spectrometry (MS).

2.8. Protein identification

The MS analyses were performed on a MALDI-TOF/TOF mass spectrometer model Ultraflex III (Bruker Daltonics GmbH, Bremen, Germany) using alpha-cyano-4-hydroxycinnamic acid as the matrix (5 μg mL^{-1} in 50% vol/vol ACN and 0.1% vol/vol TFA) in a ratio of 1:3. (sample:matrix, vol:vol). Samples were dispensed onto an MTP Anchor Chip MTP 600/384 TF target (Bruker Daltonics). The MS spectra were acquired in positive ion reflector mode, recording ions up to 3000 Da. External calibration was conducted using the Peptide Calibration Standard II (Bruker Daltonics). The equipment operates with the FlexControl 3.3 Software (Bruker Daltonics). Laser energy and the number of shots per segment spectrum were manually adjusted. After removal of interfering peaks from the matrix, which were the gel controls, trypsin and keratin, those peaks with intensity five times higher than

background in the 800 to 2000 m/z range were analyzed for protein identification, and the most intense ions were subjected to fragmentation.

The spectrum processing and database searches were performed automatically using the FlexAnalysis 3.3 Software (Bruker Daltonics) with internal calibration using the trypsin autolysis peaks. The proteins were identified using the peak lists saved as .xml and .mgf files. Searches were performed with an onsite version of the MASCOT search engine [26] (http://www.matrixscience.com/search_form_select.html) using peptide mass fingerprint (PMF) and MS/MS ion search tools, respectively, for MS and MS/MS analysis, against both the protein databases NCBInr-RefSeq [27] (http://www.ncbi.nlm.nih.gov/refseq/, Release 73) and UniProtKB/Swiss-Prot [28] (http://web.expasy.org/docs/swiss-prot_guideline.html, v. August 2015). The database search parameters included no restrictions on the protein molecular mass, maximum of one tryptic miscleavage, fixed modifications for carbamidomethylation of cysteine residues, and variable modifications for oxidation of methionine residues. The peptide mass tolerance was 0.1 Da for the precursor ions in MS spectra and 0.2 Da for the fragment ions in MS/MS spectra. Significant hits for Mascot were defined as P-value < 0.05 for peptides showing matches using PMF [29]. Additionally, a sequence coverage greater than 20% and less than 25% deviation between theoretical and experimental MW values obtained from calibrated 2D gels was defined for the identification of proteins. Protein identification was confirmed using the software BioTools, version 3.2 (Bruker Daltonics) and Mascot Daemon, version 2.3.2 (Matrix Science, UK) against a protein database for the bovidae family obtained as a subset of the UniProt database [30] (www.uniprot.org/uniprot). For endorsement of the identity of the protein, MS/MS data were evaluated by the manual *de novo* sequencing [31]. Additionally, searches in the Bovine Genome Database [32] (http://bovinegenome.org/) confirmed the identity of the proteins.

The statistical validation of protein identities was performed for score values of $P > 95\%$ in Scaffold version 3.0 (Proteome Software, EUA). The identification of proteins was verified by a manual procedure. Clustal Omega software [33] (http://www.ebi.ac.uk/Tools/msa/clustalo/, EMBL-EBI 2015) was used for alignment of MS/MS peptide ion sequences. BLAST software [34] (http://blast.ncbi.nlm.nih.gov/Blast.cgi, 2015) was used for sequence searches, PeptideCutter [35] (http://web.expasy.org/peptide_cutter/, SIB 2015) to predict potential cleavage sites by trypsin, PeptideMass [35] (http://web.expasy.org/peptide_mass/, SIB 2015) to cleave the protein sequences with trypsin and compute theoretical peptide masses, and ProtParam [35] (http://web.expasy.org/protparam/, SIB 2015) to compute physical and chemical parameters for the studied proteins.

2.9. Statistical analysis

The Statistical Analysis System Software version 9.1 [36] (http://www.ufv.br/saeg/) was used for descriptive statistical analysis. The Pearson correlation coefficient was used to determine relationships between the characteristics studied, and ANOVA and comparisons between means were performed by Tukey test at $P < 0.05$.

3. Results

3.1. Selection of semen donor bulls

The physical and morphologic characteristics of fresh (F) and frozen/thawed (FT) semen from *Bos taurus indicus* adult bulls from an AI center allowed the selection of nine Nelore bulls. The selected bulls were donors of high quality fresh (F) semen and presented variable characteristics of cryopreservation on the basis of FT semen (Table 1, Fig. 1). In F semen, the values for straight progressive sperm motility (in percentage), sperm vigor (scale from 0 to 5), and major, minor, and total spermatozoa defects (in percentage) were not different ($P < 0.05$) among the nine bulls (Table 1). However, for FT semen of the same bulls, the values for sperm motility were lower (approximately 20%–45%) than those for F semen, which was different among the animals in terms of freezability. On the basis of the variability of freezability, the bulls were ranked from 1 to 9, and it was confirmed by the adjusted trend lines (Fig. 1). The sperm vigor values were approximately 9% smaller in FT than in F semen samples, although they were similar inside each condition. Considering that BSP proteins are involved in bull semen freezability, the FT seminal plasma contents of these nine bulls were assessed individually to evaluate the differences in the quantity and composition of BSP proteins.

3.2. Protein concentration and composition in FT seminal plasma

The soluble protein concentration differed largely among the nine animals (Fig. 2A). According to the Pearson correlation test, protein concentrations were not correlated with the sperm motility of the FT seminal plasma for the nine bulls, even when three different protein determination procedures were used as follows: BCA assay (coefficient of +0.08), CBB assay (coefficient of −0.13), and CBB assay using protein samples fractionated with acetone (coefficient of +0.06). The protein concentration values ranged from 12.31 ± 0.79 to 96.9 ± 3.64 $\mu g.\mu L^{-1}$ in samples

Table 1
Physical and morphologic characteristics of fresh semen from the Nelore bulls.

Bull	Sperm motility[a,b] (%)	Sperm vigor[a] (0–5)	Major sperm defects[a] (%)	Minor sperm defects[a] (%)	Total sperm defects[a] (%)
1	70.7 ± 1.9	3.3 ± 0.3	8.4 ± 3.0	3.6 ± 1.8	12.0 ± 3.8
2	74.8 ± 2.7	3.7 ± 0.3	8.8 ± 2.5	4.0 ± 2.3	12.8 ± 4.0
3	74.0 ± 13.9	3.8 ± 0.6	7.2 ± 2.2	4.8 ± 4.0	12.0 ± 4.6
4	73.6 ± 2.4	3.6 ± 0.7	11.0 ± 3.2	3.6 ± 2.8	14.6 ± 4.9
5	72.6 ± 4.2	3.4 ± 0.4	9.9 ± 2.8	3.7 ± 1.8	13.6 ± 3.7
6	72.9 ± 3.5	3.3 ± 0.3	13.6 ± 4.4	4.4 ± 3.2	17.9 ± 6.6
7[c]	70.0 ± 0.0	3.0 ± 0.0	5.0 ± 0.0	2.0 ± 0.0	7.0 ± 0.0
8	73.5 ± 3.0	3.5 ± 0.4	10.7 ± 4.2	3.7 ± 1.8	14.4 ± 5.0
9	73.3 ± 4.1	3.3 ± 0.4	9.7 ± 2.3	4.1 ± 1.7	13.7 ± 3.7

[a] Tukey test ($P < 0.05$) showed no differences for values in each column.
[b] Straight progressive sperm motility.
[c] Results from one replicate.

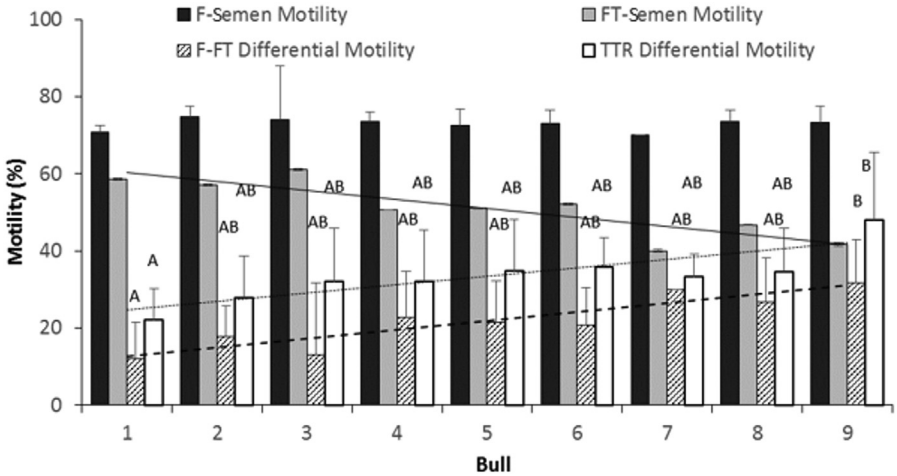

Fig. 1. Motility characteristics of fresh (F) and frozen/thawed (FT) semen from the selected Nelore bulls. The differences in motility are represented by straight progressive sperm motility of F semen (F-semen motility) and FT semen (FT-semen motility), difference in motility percent between F and FT semen (F-FT differential motility), and difference in the motility percent between F semen and the semen at the end of the thermoresistance test (TTR differential motility). A decrease in the sperm motility of the FT semen from bull 1 to bull 9 was confirmed by trend lines for FT-semen motility (___), F-FT differential motility (.....), and TTR differential motility (– – –), which had slope and r^2 values of −2.323 and +0.767, +2.295 and +0.830, and +2.167 and +0.734, respectively. The mean ± SD values were as preconized by CBRA (2013). ^{A,B}Mean values associated to the same letters do not differ by Tukey test ($P < 0.05$).

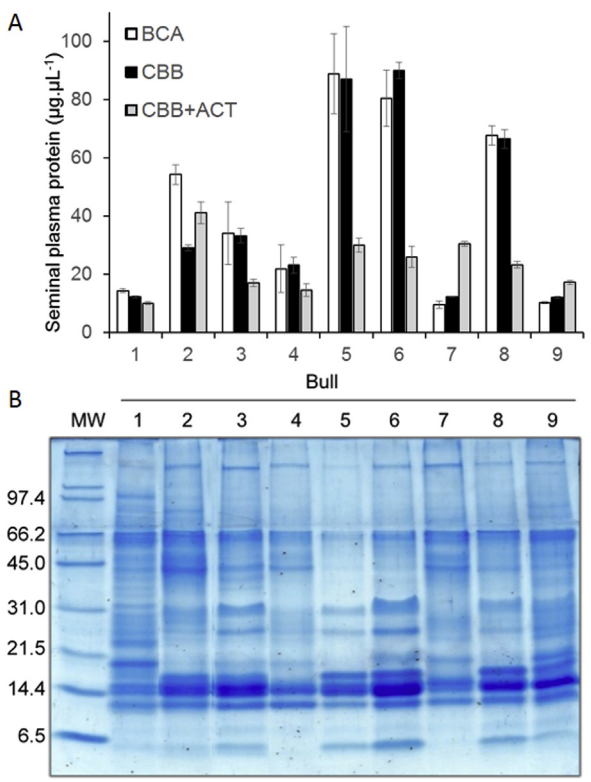

Fig. 2. (A) The soluble protein concentrations and (B) protein profile of SDS-Tricine-PAGE from the frozen/thawing (FT) seminal plasma of the nine bulls. In (A), the protein determinations were made by a bicinchoninic acid assay (BCA), a Coomassie blue assay (CBB), and a Coomassie blue assay after precipitation with pure cold acetone (CBB + ACT). In (B), 14% T gels separated 50 µg of soluble proteins in each lane, and Coomassie blue G-250 staining allowed for proteins visualization. Error bars represent the SD. Values (mean ± SD) were determined from three technical replicates in three FT samples for each animal. MW, molecular weight.

not treated with acetone using the CBB method, which is higher than values determined for samples treated with acetone, 10.08 ± 0.41 to 39.91 ± 1.26 $\mu g.\mu L^{-1}$.

Electrophoresis by SDS-Tricine-PAGE revealed a wide variety in the protein composition and individual protein concentrations in the seminal plasma from the nine bulls. Proteins smaller than 30 kDa were abundant in all samples (Fig. 2B). Consequently, 2-DE using a 14% T gel in the second dimension was completed.

3.3. Two-dimensional electrophoresis with a pH range from 3 to 10

The protein profiles of FT seminal plasma differed for the nine bulls in the abundance and types of proteins in 2-DE gels. Four protein spots of 15 to 20 kDa had raised our interest (highlighted in Fig. 3). The spots are called S1, S2, S3, and S4 from this point of the work. These spots showed up isolated and were identified in the gels of bulls 1, 2, 3, 4, 8, and 9, but they did not occur in the gels of bulls 5 and 6. In addition, only one replicate of the motility data in F semen of bull 7 was available, and bulls 5, 6, and 7 were not considered for the next analyses. The linear statistical model also explains the reduction in freezability for FT semen for the bulls 1, 2, 3, 4, 8, and 9, as it is similar to that shown for the nine bulls in Figure 1. Values of r2 for the trend lines were 0.928, 0.921, and 0.884 for FT-semen motility, F-FT differential motility, and TTR differential motility, respectively.

The comparative abundance of the proteins, quantified by the volume of spots, showed that the spot S4 was absent in all replicates of gels from bulls 1, 2, and 3, and that a high abundance of spot S4 was found in gels from bulls 4, 8, and 9 (Fig. 4). Moreover, the calculated Pearson correlation coefficients indicated negative and high correlations for sperm motility in the FT semen with spot S4 volume and with the major and total spermatozoa defects. Additionally,

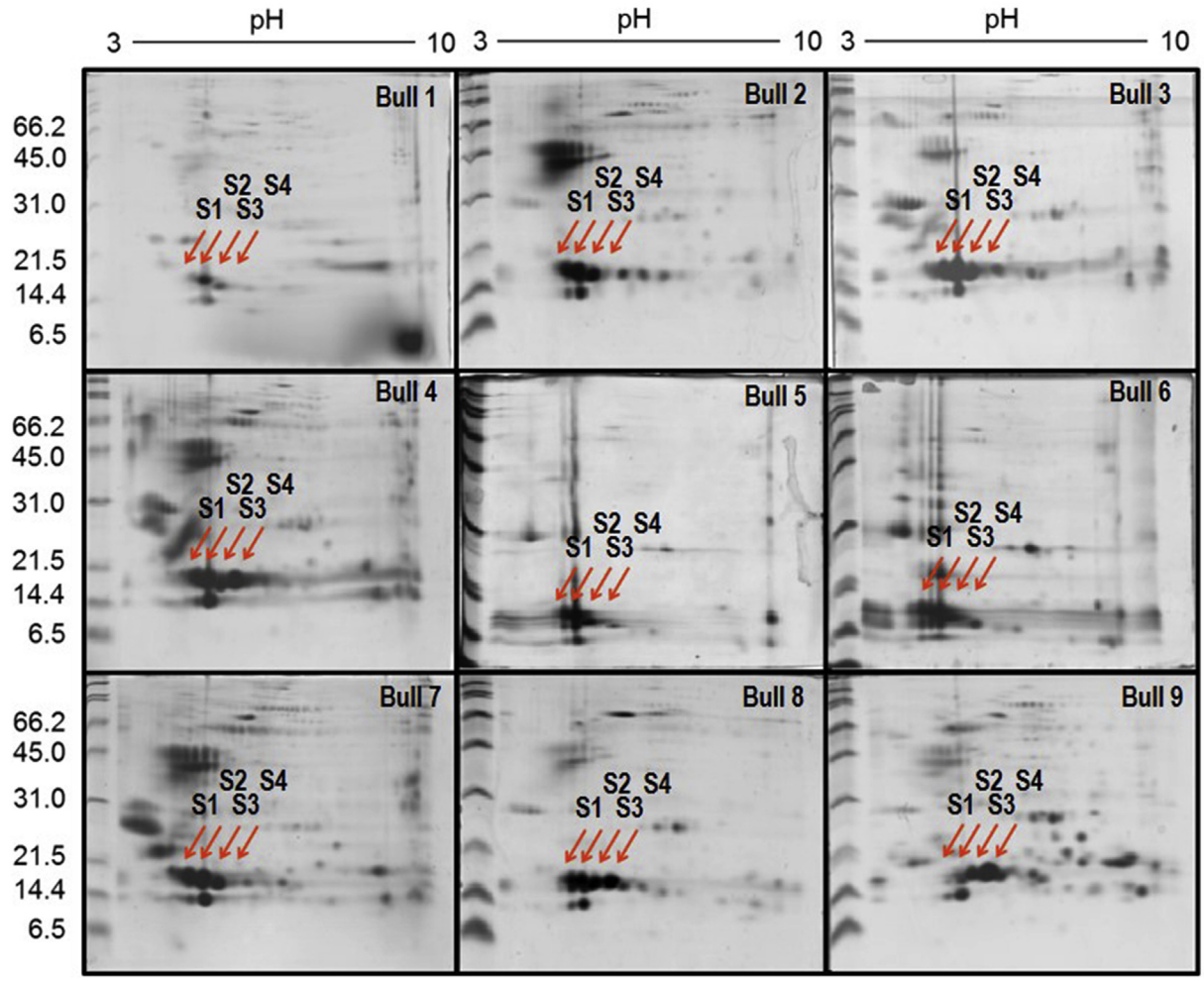

Fig. 3. Two-dimensional protein profiles of the frozen/thawing seminal plasma of the nine bulls (bull 1–bull 9). Linear pH 3 to 10 IPG strips and 14% T SDS-Tricine-PAGE separated 120 μg of soluble proteins on each gel. The spots highlighted by arrows corresponded to the interesting spots S1 to S4. Coomassie blue G-250 staining allowed proteins visualization.

the Pearson coefficient showed a positive, high correlation of this sperm motility with sperm vigor, suggesting the involvement of the protein in spot S4 with low semen freezability (Table 2).

3.4. Identification of BSP1 using 2-DE on pH 3 to 10 IPG strips

The PMF using the Mascot search engine was not capable of identifying the proteins in spots S1, S2, S3, and S4 with $P < 0.05$, despite the high intensity of the spots in the MS spectra (Fig. 5A). A small number of peptide fragments from 1000 to 3000 Da were produced (Fig. 5A). Peptide fragment fingerprinting (PFF) and *de novo* sequencing allowed the identification of the protein BSP1 (SFP1_BOVIN, UniProtKB ID P02784) in these four spots for all assessed bulls. The total similarity of the amino acid sequence for the ions 1187.622 Da and 2739.957 Da (Fig. 5B) was detected. Analysis of the theoretical cleavage sites for trypsin in the BSP1 sequence (UniProtKB ID P02784) using the PeptideCutter software (Supplementary Table 1) showed the occurrence of 12 theoretical cleavage sites for trypsin in the protein, which produced few peptides analyzable by MS, preventing their identification. These theoretical cleavage sites are close to the carboxy-terminus of the residues 56, 58, 59, 82, 84, 89, 93, 103, 104, 110, 129, and 132 (Fig. 5C). The lysine residue (K) 127 is not a true cleavage site because this K is bordered by an aspartic acid residue (D) [37].

Proteins in spots S1 to S4 were consistent with the MW and isoelectric point (pI) medium values of BSP1, which were 17.24 ± 1.87 kDa and pI 4.69 ± 0.11 (spot S1); 16.75 ± 1.13 kDa and pI 4.98 ± 0.10 (spot S2); 16.30 ± 0.82 Da and pI 5.31 ± 0.09 (spot S3); and 16.65 ± 1.31 kDa and pI 5.70 ± 0.08 (spot S4). The means ± SD was obtained from the 27 gels of the nine bulls and three gel replicates.

3.5. Identification of BSP1 using 2-DE on pH 4 to 7 IPG strips

Separation of the FT seminal plasma proteins from bulls 1 and 8 by 2-DE using pH 4 to 7 IPG strips confirmed the

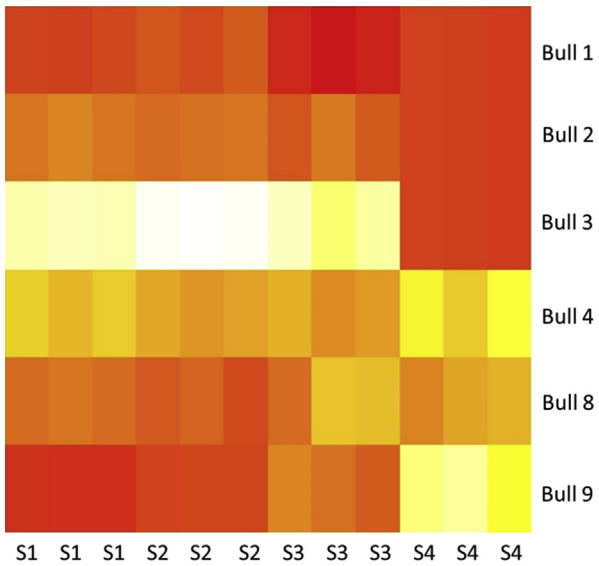

Fig. 4. Differential abundances of the spots S1 to S4, identified as Binder of SPerm 1 forms, obtained from the two-dimensional protein profiles of the frozen/thawed seminal plasma of six bulls (bull 1, 2, 3, 4, 8, and 9) in three replicates of the gels. In the heatmap, dark squares represent low protein abundance, and light squares represent high protein abundance. The spot-S4 abundance increased from bull 1 to bull 9.

identity of BSP1 in the spots S1, S2, S3, and S4 (Fig. 6). The two-dimensional protein profiles were similar to those obtained with the pH 3 to 10 IPG strips (Fig. 3). However, seven spots (A–G) were separated. Comparison of Figure 3 (spots S1–S4) and Figure 6 (spots A–G) shows that the spots S2 and S3 were both divided into two spots, producing the spots B/C and D/E, respectively. The spot A was then assessed. Despite the small number of ions from 1000 to 3000 Da produced in the MS spectra for BSP1 (Supplementary Table 1), the spots C, D, F, and G (Fig. 6) were identified as BSP1 ($P < 0.05$; Table 3). They correspond to the spots S1, S2, S3, and S4, respectively (Fig. 5).

The spots H and I were identified as spermadhesin-1 protein (SPAD1_BOVIN, UniProtKB ID P29392) from *Bos taurus* with experimental values of 11.75 ± 1.39 kDa and pI 4.90 ± 0.07 for spot H and 11.73 ± 1.36 kDa and pI 5.17 ± 0.08 for spot I (the mean ± SD values obtained for the 27 gels). Spot J corresponded to a clean piece of gel that worked as a negative control (Table 3). The protein in spot E was identified by MS spectra analysis as an acylphosphatase-1 (ACYP1_PIG) from *Sus scrofa* (wild boar) (UniProtKB ID P24540; $P < 0.05$; Table 3). Comparing ACYP1_PIG from *Sus scrofa* with acylphosphatase-1 (ACYP1_BOVIN) from *Bos taurus* (bovine; UniProtKB ID P41500), the sequence identity of the amino acid sequence was 94.06%. Positivity was found for four of the six nonidentical residues (obtained by hand). The proteins present in spots A and B were not identified ($P < 0.05$) by the procedures used in this work.

Table 2
The Pearson correlation coefficients among characteristics of the fresh (F) and frozen/thawed (FT) semen from the bulls 1, 2, 3, 4, 8, and 9.

Characteristics	Sperm motility in the FT semen[a]	Spot S4 volume[a]
Major spermatozoa defects in F semen	−0.75	+0.79
Total spermatozoa defects in F semen	−0.79	+0.84
Sperm vigor in FT semen	+0.85	−0.90
Spot S4 volume	−0.96	+1.00

[a] (+) and (−) signals mean positive and negative Pearson correlation between characteristics.

The medium values of the molecular masses and the pI values of the spots A to G are consistent with BSP1, which were 15.93 ± 0.42 kDa and pI 4.74 ± 0.08 (spot A), 15.96 ± 0.34 kDa and pI 4.91 ± 0.06 (spot B), 15.76 ± 1.55 kDa and pI 4.92 ± 0.10 (spot C), 15.98 ± 0.50 kDa and pI 5.15 ± 0.08 (spot D), 14.89 ± 0.51 kDa and pI 5.14 ± 0.08 (spot E), 15.14 ± 0.58 kDa and pI 5.43 ± 0.08 (spot F), and 15.52 ± 0.53 kDa and pI 5.78 ± 0.12 (spot G). Moreover, a meticulous analysis of the MS profiles showed that six ions were present in the spectra from the spots A to G (Supplementary Fig. 1). The ions 2333 and 2867 Da that were not found in the profile of spot B (Table 4) were also not found in spot E. Five of these ions, with m/z-values of 2097, 2154, 2333, 2739, and 2867, were sequenced by a *de novo* procedure and by the software BioTools using the MS/MS spectra. Searches in the Bovine Genome Database confirmed that these sequences corresponded to ions with m/z-values belonging to the BSP1 sequence, with high score values and errors smaller than 10^{-5}, and total identity and positivity (Table 4). For spots A and B, however, additional information is necessary for securely assuming that they correspond to forms of the BSP1 protein, even though the MS spectra of these spots showed ions consistent with BSP1 (Supplementary Fig. 1).

Spots C, D, F, and G were confirmed and validated as the BSP1 protein ($P < 0.05$), and spot G (which is the spot S4) presented a higher abundance in cryopreserved seminal plasma of Nelore bulls with the lowest freezability of the semen, whereas it was absent in bulls with the highest freezability.

4. Discussion

The BSP1 protein has been described as a double-edged sword, as it is both beneficial and unfavorable to sperm fertility and freezability [38]. Our results showed the presence of more than two forms of BSP1 in FT bovine seminal plasma of Nelore bulls. However, not all four forms of BSP1 identified were present in the nine assessed breeding bulls from the AI center, suggesting the involvement of different forms of BSP1 in the cryopreservation ability. In particular, the spot weighing 15.52 ± 0.53 kDa with a pI 5.78 ± 0.12 was abundant in bulls with the lowest freezability of the semen, whereas it was absent in bulls with the highest freezability.

BSP proteins have been found to be involved in the variability of the freezability of semen from donor bulls [6,38,39]. Studies on BSP1 function have investigated the general involvement of the protein after purification as a glycoprotein, without assessing the composition and the role of different forms of BSP1. Works about the biochemical and functional properties of this protein describe BSP1 as a

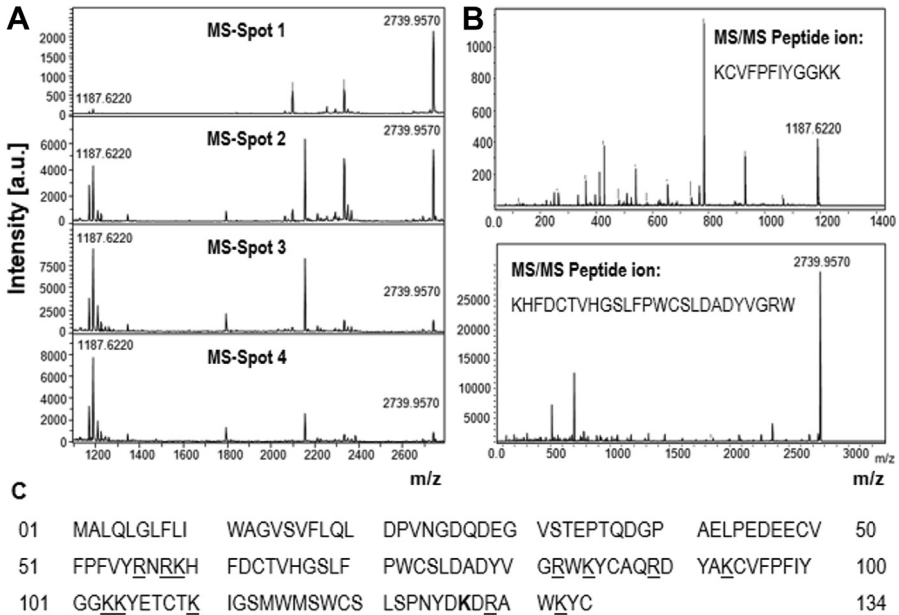

Fig. 5. (A) Mass spectrometry (MS) profiles of the spots S1 to S4, (B) the ion fragmentation (MS/MS) profiles for the ions 1187.6 and 2739.9 Da, which are present in spots S1 to S4, and (C) the 12 theoretical cleavage sites by trypsin (R or K residues underlined) in the sequence of the Binder of SPerm 1 protein precursor. K127 (bold type) is not a cleavable site used by trypsin.

multifunctional, polydisperse, multimeric self-associated molecule. BSP1 can act by enhancing sperm motility and inducing sperm capacitation and the acrosome reaction, but it also causes increases in cholesterol efflux from spermatozoa, inducing injury to the sperm during cryopreservation [3,11,12,40]. The participation of BSP1 in semen fertility and freezability is not completely understood [41], and information about the mechanisms of action and the regulation of BSP1 action is not conclusive [39]. Consequently, different forms of BSP1 could potentially be involved in different steps of the physiological mechanisms related to reproduction and cryopreservation.

The primary chain of the BSP1 protein has been considered unique for almost 30 years [9,42], with the protein showing only differences in its glycosylation. However, a recent study using Fourier transform ion cyclotron resonance MS has shown four sequence variants for the BSP1 protein [43]. Concisely, one variant contained point mutations (P10L and G14R), one had a 14-residue truncation in the N-terminal region, and two variants were identified

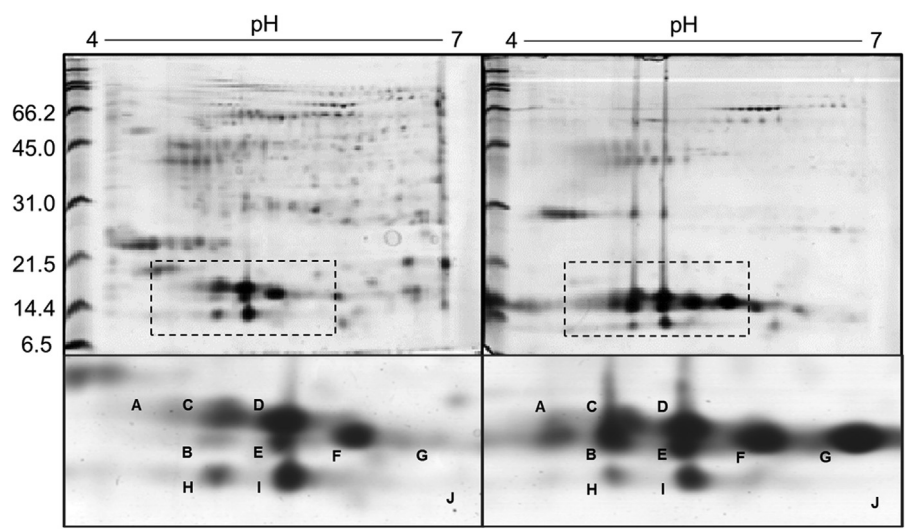

Fig. 6. Two-dimensional protein profiles (above) and the highlights of the protein spots from A to J in the gels (below) of frozen/thawed seminal plasma from bull 1 (left) and bull 8 (right). Linear pH 4 to 7 IPG strips and 14% T SDS-Tricine-PAGE separated 120 μg of soluble proteins in each gel. The spots of interest were named A to G. The spots H and I worked as positive control, and spot J was a negative control for protein identification.

Table 3
Identification of proteins in the spots A to J in the two-dimensional gels of frozen/thawed seminal plasma from bull 1 and bull 8 by MS spectra in MALDI-TOF/TOF using the MASCOT search engine (www.matrixscience.com).

Spot	Accession[a]	Protein description (organism)	MW (Da) (theor/exper)[b]	pI value (theor/exper)[b]	MS protein score (expect)	Sequence covered (%)	Matches	Database
A	No protein identified	—	n.i./15,927	n.i./4.75	—	—	—	UniProtKB/Swiss-Prot; NCBInr
B	No protein identified	—	n.i./15,964	n.i./4.91	—	—	—	UniProtKB/Swiss-Prot; NCBInr
C	SFP1_BOVIN (UniProtKB ID P02784)	Binder of SPerm1 (BSP1) protein (*Bos taurus*)	15,926/15,755	4.91/4.92	63/54	32	5/19	UniProtKB/Swiss-Prot
D	SFP1_BOVIN (UniProtKB ID P02784)	Binder of SPerm1 (BSP1) protein (*Bos taurus*)	15,926/15,976	4.91/5.15	67/54	39	8/29	UniProtKB/Swiss-Prot
E	ACYP1_PIG (UniProtKB ID P24540)	Acylphosphatase-1 (*Sus scrofa*)	11,420/14,887	7.98/5.14	61/54	70	4/24	UniProtKB/Swiss-Prot
F	SFP1_BOVIN (UniProtKB ID P02784)	Binder of SPerm1 (BSP1) protein (*Bos taurus*)	15,926/15,145	4.91/5.43	69/54	32	7/21	UniProtKB/Swiss-Prot
G	SFP1_BOVIN (UniProtKB ID P02784)	Binder of SPerm1 (BSP1) protein (*Bos taurus*)	15,926/15,517	4.91/5.78	69/54	32	7/22	UniProtKB/Swiss-Prot
H	SPAD1_BOVIN (UniProtKB ID P29392)	Spermadhesin-1 (*Bos taurus*)	15,036/11,749	5.07/4.90	63/61	52	7/19	UniProtKB/Swiss-Prot
I	SPAD1_BOVIN (UniProtKB ID P29392)	Spermadhesin-1 (*Bos taurus*)	15,036/11,730	5.07/5.17	72/61	64	8/28	UniProtKB/Swiss-Prot
J	Negative control (clear gel)	—	—	—	—	—	—	UniProtKB/Swiss-Prot; NCBInr

Abbreviations: BSP1, Binder of SPerm 1; MW, molecular weight; n.i, not identified; pI, isoelectric point.
[a] Protein identification was according Tukey test at $P < 0.05$.
[b] Identification of spermadhesin-1 worked as a positive control to confirm the procedure.

after affinity purification. The authors conclude that BSP1 is naturally produced as a mixture of several protein forms that are not yet studied. Considering protein identification is typically carried out by MS procedures, variations in the primary structure of BSP1 are not detected unless the protein is partially or completely sequenced. Variances in the amino acid sequence may produce multiple forms of BSP1 with differences in mass and pI values.

Variances in the glycosylated portion of BSP1 may also be responsible for the structural and functional properties of the forms of BSP1. Glycosylation has been extensively studied over the past two decades as the most common covalent protein modification influencing functional processes in eukaryotic cells [15,44]. BSP1 has been studied as a heparin-binding protein [3,39], which is generally purified by affinity chromatography for the unspecific glycosylated portion of the molecule, not allowing the separation of the forms of BSP1. A recent study revealed that sequestration of BSP1 from semen samples led to a significant improvement in the cryopreservation of

Table 4
Ions with isotopic patterns in the mass spectrometry profiles of the spots A to G in two-dimensional gels of the seminal plasma from the bull 8.

Ions with isotopic patterns in MS profile (Da)	Position of cleavage sites	Resulting peptide sequence	Spectrum containing the peptide mass[c]							Protein identification[b,d]
			A	B	C	D	E	F	G	
2097.020 and 2154.000[a]	39–56	GPAELPEDEECVFPFVYR	X	X	X	X	x	X	X	ref|NP_001001145.1 Seminal vesicle secretory protein 109; score: 44; E-value: 10^{-5}
2867.240[a]	59–82	KHFDCTVHGSLFPWCSLDADYVGR[a]	x	-	X	X	-	X	X	ref|NP_001001145.1 Seminal vesicle secretory protein 109; score: 60; E-value: 2×10^{-10}
2739.083[a]	60–82	HFDCTVHGSLFPWCSLDADYVGR	X	x	X	X	x	X	X	ref|NP_001001145.1 Seminal vesicle secretory protein 109; score: 58; E-value: 8×10^{-10}
2333.075[a]	111–129	IGSMWMSWCSLSPNYDKDR	x	-	X	X	-	X	X	ref|NP_001001145.1 Seminal vesicle secretory protein 109; score: 48; E-value: 7×10^{-7}
2797.000	n.i.	n.i.	X	X	x	x	X	X	X	n.i.

Abbreviation: n.i., not identified.
[a] Alkylation with iodoacetamide adds 57.05 Da in the cysteine residues.
[b] Identification using the Bovine Genome Database [32], BlastP software [34], and Bovine NCBI RefSeq Proteins Database [27].
[c] The capital letter "X" indicates high ion abundance in the spectra, and lowercase letter "x", low abundance; the hyphen indicates the absence of the ion.
[d] Identity of 100% for all identified ions.

Crossbred bull spermatozoa [3] and that heparin-binding proteins and BSP1 in nonfreezable semen might be responsible for many cryoinjuries [39].

Proteomics using 2-DE is appropriate to isolate and identify the forms of the BSP proteins because these protein forms present small variances in mass and pI values, even when they are produced by post-translational modifications or changed by proteolysis [15,41]. For BSP1, which is reported as an exclusively monomeric protein at low concentrations and may form weakly bound oligomers [43], the 2-DE reagents induce disaggregation to allow the individual study of the forms of the protein.

Proteomics of bovine seminal plasma using 2-DE have been used to study proteins involved in freezability. However, the results suggest difficulties in the identification of forms of BSP1 from gels using traditionally used procedures. Jobin et al. [45] analyzed 16 *Bos indicus* bulls and identified 12 proteins. Three were found in all animals, but they did not safely identify proteins in spots in positions similar to the forms of BSP1. Rego et al. [46] identified four spots in *Bos indicus* bulls similarly with the BSP5-like precursor from *Ovis aries*, with a size and pI in the range of BSP1. The authors also identified six spots as BSP1 with a mass and/or pI higher than those reported for BSP1 with low sequence coverage (7 or 21%). The differences were not discussed. Aslam et al. [47] used difference gel electrophoresis to evaluate crossbred bulls and observed the differential abundance of proteins in a position equivalent to the BSP1 forms identified in this work. However, they did not identify the proteins. Thus, BSP1 is not easy to identify, and structural and chemical information about the molecule is required for a safe identification.

The high similarity of the mass and pI values of the BSP1 and BSP3 proteins may yield questions about the identification as BSP1 of the four spots. BSP3 (SFP3_BOVIN, UniProtKB ID P04557) is also a member of the BSP proteins in bovine animals. The BSP3 sequence is 67.16% identical to BSP1, weighs approximately 15.0 to 16.5 kDa, and its pI value ranges from approximately 5 to 5.5 [9,13,48–51]. In fresh bovine seminal plasma, BSP1 protein represents approximately 25 to 47% of the total protein content, whereas BSP3 represents, at most, 7% [10,52]. In this study, we assessed the characteristics of BSP3 and identified proteins that did not correspond to BSP3. BSP3 ions were not detected in the obtained spectra. The cleavage positions for theoretical proteolysis with trypsin using PeptideCutter differ in the sequences of BSP1 and BSP3. In addition, the high abundance of proteins in the identified spots strengthens the identity of BSP1.

The inverse correlation suggested between spot G (or spot S4) and freezability aroused our interest because this form of BSP1 may be evaluated as a valuable tool for the study of the cryopreservation of semen to understand the occurrence of low freezability and for selecting bulls with high freezability for commercial uses. Routine andrological and complementary tests in bull semen as recommended by CBRA [4] are not sufficient to predict the potential of the cryopreservation of semen [53–56] or show large ranges of variation [53]. The reported Pearson correlation values involving motility and fertility have ranged from as low as 0.15 to as high as 0.83 [55,57,58]. Researchers continue to search for new methodologies for selecting high-quality bulls [3,12,38,39].

Bulls belonging to AI centers are carefully selected by presenting semen with high quality and freezability for commercial purposes. For this work, the detection of bulls with variable patterns of freezability from an AI center was not an easy task. The accurate obtainment of the nine assessed bulls [4], which were ranked from 1 to 9 on a scale for the decrease in freezability, offered appropriated samples for comparative proteomics. Proteomics is a time-consuming and costly procedure. The individual evaluation of the nine bulls in three replicates required hard work to perform and analyze the gels by MS and bioinformatics.

The evaluation of single semen samples from individual bulls, as used in this study, was also a powerful and fundamental tool to identify variations in freezability. Semen collected from different animals or collected from the same animal at different moments may be influenced by factors such as the physical conditions at which these animals are maintained, seasonality, and the frequency of semen collection [59,60]. Our results using 2-DE have been directly correlated with the andrological results of semen for a particular donor bull from the AI center, which had already been preselected as presenting low freezability.

Three valuable findings have been obtained in this study, which are (1) the identification of four forms of the BSP1 protein in the seminal plasma of breeding bulls; (2) the differential abundance of the forms of BSP1 among animals presenting semen with different patterns of freezability; and (3) the inverse correlation observed between spot G (or spot S4) abundance and the freezability of the bovine semen.

Further studies about forms of BSP1, including that present in spot G (or spot S4), are necessary to study and unveil correlations among BSP1 and events related to semen freezability in bulls.

4.1. Conclusions

BSP1 proteins in Nelore bulls were identified as a group of at least four forms of the protein that could be individually involved in different physiological events related to reproduction and the cryopreservation of the semen. The identification of forms of the BSP1 protein is not easy and requires a combination of techniques of MS and bioinformatics. The form of BSP1 protein corresponding to the spot weighing 15.52 $\pm$ 0.53 kDa with a pI 5.78 $\pm$ 0.12 (spot G or S4), which showed high abundance in bull semen with low freezability, must be studied to identify a low-freezability predictor of sperm. A single evaluation of the semen samples of each bull showed to be essential for studying the relationship among the abundance of the forms of BSP1 and the freezability. Two-dimensional electrophoresis is a rapid and high-resolution tool to identify forms of BSP1 proteins in bull semen. The 2-DE procedure seems appropriated to be used as a routine tool in laboratories to aid the selection of bulls and evaluate the semen samples for assisted reproduction.

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Authors' contributions: M.J.M.J. performed the biochemical analyses of the seminal plasma including protein determination, protein separation by two-dimensional electrophoresis, and protein identification by mass spectrometry analysis. L.F.M. performed the collection, treatment, cryopreservation, and physical and morphologic evaluations of the semen; prepared the seminal plasma for cryopreservation. R.L.S. participated in activities regarding mass spectrometry analysis and protein identification. T.F.S. participated in activities regarding protein separation by two-dimensional electrophoresis. D.S.O. participated in activities regarding protein determination and isolation in one-dimensional gel electrophoresis. P.R.G.P. led discussions on physiological correlations of the biochemical results, performed correlations analyses. P.R.G.P., A.F.-C., S.V.A.C., J.D.G., and M.C.B.-P. reviewed and edited the article. A.F.-C. and S.V.A.C. contributed in performing the computational analyses. J.D.G. conceived the idea; maintains collaboration with the AI Center; selected the bulls for obtainment of semen, advised and supervised activities regarding collection, treatments, cryopreservation and physical and morphologic evaluations of the semen; performed statistical analyses of the andrological parameters evaluated. M.C.B.-P. designed and supervised activities of work regarding biochemical analysis of the seminal plasma; performed the computational analyzes for validating protein identity; drafted the article. All authors have read and approved the final article.

Competing Interests

The authors have nothing to disclose as conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at http://dx.doi.org/10.1016/j.theriogenology.2016.02.030.

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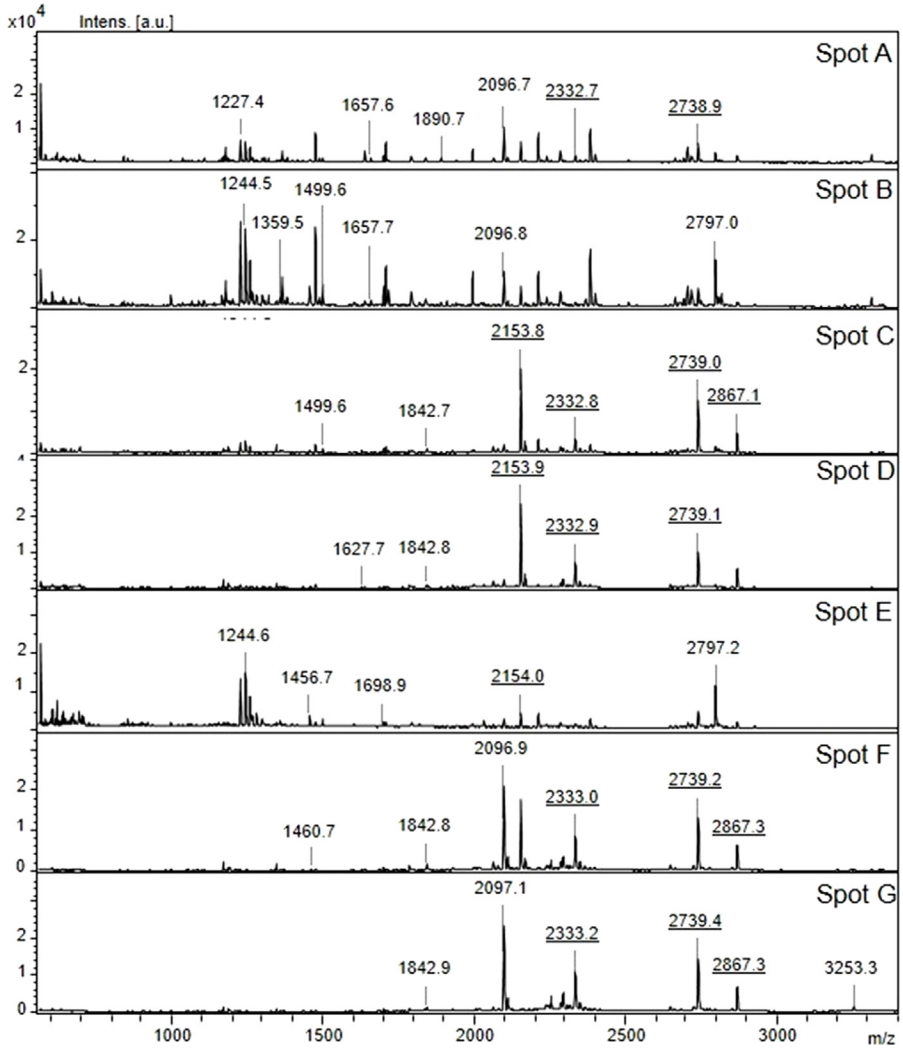

Supplementary Fig. 1. Mass spectrometry profiles of the spots A to G in two-dimensional gels of the frozen/thawed seminal plasma from bull 8. The ions 2333, 2097, 2154, 2739, 2797, and 2867 Da were present in the spectra from the spots A to G. The ions 2333 and 2867 that were not found in the profile of spot B were also not found in spot E (that is an acylphosphatase-1). Five of these ions were sequenced by a *de novo* procedure and using the BioTools software on the basis of the MS/MS spectra (Table 4). Searches in The Bovine Genome Database confirmed the identity as a BSP1.

Supplementary Table 1
Theoretical fragmentation of the sequence of BSP1 by trypsin using PeptideCutter software considering the possibility of one missed cleavage.

Position of cleavage site[a]	Resulting peptide sequence	Peptide mass [Da] [M]	Peptide mass [Da] [M+1]	Number of Cys (alq)[b]	Mass [M+1] and Cys (alq)	Mass [M+1] and Cys (alq) −18 Da water
1–56	MALQLGLFLIWAGVSVFLQLDPVNGD QDEGVSTEPTQDGPAELPEDEECVFPFVYR	6183.909	6184.909	1	6241.959	6223.959
39–56	GPAELPEDEECVFPFVYR	2097.300	2098.300	1	2155.350	2137.350
57–58	NR	288.307	289.307	0	289.307	271.307
59	K	146.189	147.189	0	147.189	129.189
60–82	HFDCTVHGSLFPWCSLDADYVGR	2625.916	2626.916	2	2741.016	2723.016
59–82	KHFDCTVHGSLFPWCSLDADYVGR	2772.105	2773.105	2	2887.205	2869.205
83–84	WK	332.403	333.403	0	333.403	315.403
85–89	YCAQR	639.727	640.727	1	697.777	679.777
90–93	DYAK	495.533	496.533	0	496.533	478.533
94–103	CVFPFIYGGK	1130.370	1131.370	1	1188.420	1170.420
104	K	146.189	147.189	0	147.189	129.189
105–110	YETCTK	743.830	744.830	1	801.88	783.88
111–129	IGSMWMSWCSLSPNYDKDR	2276.584	2277.584	1	2334.634	2316.634
130–132	AWK	403.481	404.481	0	404.481	386.481
133–134	YC	284.330	285.330	1	342.38	341.38

Five different peptide ions may be theoretically generated from the cleavage by trypsin, considering no missed cleavage.
[a] Close to the carboxi-terminus of the residues 56, 58, 59, 82, 84, 89, 93, 103, 104, 110, 129, and 132.
[b] Add 57.05 Da.