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Identification of RSVP14 and RSVP20 Components by Two-dimensional Electrophoresis and Western-blotting

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Contents

We have already shown that RSVP14 and RSVP20, two ram seminal plasma (SP) proteins postulated to be involved in sperm capacitation and gamete interaction can protect spermatozoa against cold-shock. In this study, we use two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for the analysis of SP proteins of Rasa Aragonesa rams, using enhanced protein solubilization in the presence of tributyl phosphine (TBP) and a polyacrylamide linear gradient gel with a narrow pH range (4–7). The image analysis of the 2D map detected 195 protein spots, with isoelectric points (pIs) ranging from 4.5 to 6.6, and molecular weight (M_r) from 11.7 to 90.4. Staining of 2D gels with Pro-Q Emerald 300 Glycoprotein Stain revealed that most significant proteins in ram SP are glycosylated. The removing of protein N-linked oligosaccharides improved the gel resolution. 2D-PAGE analysis of the whole fraction 6 (F6) separated from ram SP by exclusion chromatography showed six main protein spots, four (*a*, *b*, *c*, *d*) in the 14 kDa and two (*e*, *f*) in the 20 kDa region. Western-blot analyses indicated that the anti-P14 antibody recognized four spots on the SP map, 4, 5, 6 and 7, that matched with spots *a*, *b*, *c*, *d* of F6 map. The anti-P20 antibody recognized spots 13 and 14 of SP map that corresponded to spots *e*, *f* of F6 map. The deduced sequences by *de novo* sequencing evidenced that protein spots 7 and 13 have significant similarities to BSP family, while protein spots 4 and 14 did not appear to be homologous with any reported protein in the current mammalian Proteinbank databases.

Introduction

Mammalian seminal plasma (SP), a physiological secretion from multiple glands of the male reproductive tract, functions as a vehicle for ejaculated spermatozoa (Mann 1978) and is important for sperm function and survival (Edwards et al. 1981). Several studies have provided direct evidence that specific components of SP, particularly proteins, are adsorbed onto the surface of ejaculated sperm (Fuller et al. 1994; Watson 1995; Perez et al. 1996; De Jonge 1999). Some of these adsorbed proteins maintain the stability of the membrane until the process of capacitation (decapacitation factors) in the female genital tract (Manjunath et al. 1993; Fraser et al. 1996; Ollero et al. 1996), when their removal is a prerequisite for fertilization (Manjunath et al. 1993; Yanagimachi 1994; Fraser et al. 1996; Perez et al. 1996).

Certain SP proteins have been described as infertility factors (Brandon et al. 1999) while other heparin-binding proteins have been associated with sperm fertility (Miller et al. 1990). In addition, the need for some of these adsorbed proteins, not only to acquire the fertilizing capacity, but also to maintain cell viability has already been reported (Harrison and Vickers 1990; Brandon et al. 1999). Likewise, we have already shown that ram SP proteins can repair (Barrios et al. 2000) and prevent (Pérez-Pé et al. 2001a,b) cold-shock sperm membrane damage, and that seasonal differences in ram SP proteins could affect their ability to recover membrane integrity of cold-shocked sperm (Pérez-Pé et al. 2001a,b). Very recently, we have proved that two ram SP protein bands of approximately 14 and 20 kDa (Barrios et al. 2005), called RSVP14 and RSVP20, respectively, as they are exclusively synthesized in the seminal vesicles (Fernández-Juan et al. 2006), are responsible for this protective effect.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used for the separation and characterization of several proteins from SP of bull (Desnoyers et al. 1994; Mortarino et al. 1998; Jobim et al. 2004; Moura et al. 2006), ram (Souza et al. 2004; Jobim et al. 2005), horse (Brandon et al. 1999), boar (Sanz et al. 1993) and human (Bohring et al. 2001). As the use of a polyacrylamide linear gradient gel should increase the analysis resolution, the aim of this study was to improve the polypeptide detection in the SP 2D-map of Rasa Aragonesa rams, using enhanced protein solubilization in the presence of tributyl phosphine (TBP), and a polyacrylamide linear gradient gel with a narrow pH range (4–7). Moreover, we identified the protein components of RSVP14 and RSVP20 (Fernández-Juan et al. 2006), two ram SP protein bands postulated to be involved in sperm capacitation and gamete interaction (Barrios et al. 2005), by performing western-blot analysis for specific detection of both proteins.

Materials and Methods

Semen collection and processing

All the experiments were performed with fresh semen taken from May 2003 to April 2004 from nine mature Rasa Aragonesa rams using an artificial vagina. All the rams belonged to the National Association of Rasa Aragonesa Breeding (ANGRA) and were 2–4 years old. They were housed at the animal experimentation service of the University of Zaragoza under uniform nutritional conditions. Based on the positive results from a previous study, sires underwent an abstinence period of 2 days, and second ejaculates were pooled and used for each assay, to avoid individual differences (Ollero et al. 1996).

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Collection of seminal plasma

Seminal plasma was obtained by spinning 1 ml of semen at $7500 \times g$ for 5 min in a microfuge at 4°C. The supernatant was centrifuged again, SP was recovered and, after filtering through a 0.45 μm Millipore membrane (Millipore Ibérica, Madrid, Spain) and adding 10% of a protease and phosphatase inhibitor cocktail (Sigma Chemical Co., St Louis, MO, USA), was kept at −20°C. The protein content was determined using Bradford's method (Bradford 1976).

2D-electrophoresis

Seminal plasma samples were prepared for electrophoresis as follows: 75 μg of SP and 50 μg of F6 (fraction 6 of SP by exclusion chromatography on Sephacryl-100 HR; Barrios et al. 2000) proteins were diluted in 125 μl of sample buffer containing 8 M urea, 2% [3-(3-(cholamydopropyl) dimethyl-ammonio)-1 propane sulphonate] (CHAPS), 40 mM DL-Dithiothreitol (DTT), 0.2% Bio-Lyte™ 3/10 ampholyte (Bio-Rad, Hercules, CA, USA), 0.0002% Bromophenol Blue, 2 μl of TBP commercial solution (Sigma Chemical Co.) and 5 μl 2D-PAGE standards (Sigma Chemical Co.).

Samples were subjected to the 2D-PAGE as described by O'Farrel et al. (O'Farrel et al. 1977). Proteins were separated by isoelectric focusing (IEF) using 7 cm immobilized pH gradients strips (IPGs; pH 4–7; Bio-Rad). The IPGs were placed overnight in the channel of a rehydration tray that contained the solution described above. Isoelectric focusing was performed using a Protean® IEF Cell (Bio-Rad) at 8250 V and 20°C. After isoelectrofocusing, the IPG strips were equilibrated for 10 min in 2500 μl of equilibration buffer I containing 6 M urea, 375 mM Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2% (w/v) DTT, and 10 min longer in 2500 μl of equilibration buffer II containing 6 M urea, 375 mM Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) iodoacetamide. Finally, the second-dimension run was performed on a 9–20% polyacrylamide linear gradient gel (SDS-PAGE), using a Miniprotean II (Bio-Rad).

After electrophoresing in the second dimension, SP gels were stained with Sypro-Ruby Protein Gel Stain (Molecular probes, Barcelona, Spain) and F6 gels with 0.025% Coomassie R (Serva, Heidelber), and scanned with a gel doc System with Molecular Analyst software (Bio-Rad). The 2D gel images were processed for analysis with PD-Quest™ 2-D analysis software (Bio-Rad) to determine the relative protein content of the spots. We used a correlative numbering system according to molecular weight (M_r) to designate spots. Four gels of SP were made per month. The relative amount of each spot was assessed as the percentage, and results are shown as the mean (±SEM) of the four samples indicated in each case.

Western-blotting

The 2D gels were transferred for 1 h onto a PVDF-membrane with a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Non-specific sites on the membranes were blocked for 1 h with 5% BSA in blocking buffer (Tris-HCl 10 mM pH 8; NaCl 120 mM, 0.05% Tween 20). The proteins were immunodetected by incubating for 3 h at room temperature with the anti-P14 (1 : 4000) or P20 (1 : 750) polyclonal antibodies (Barrios et al. 2005) diluted in blocking buffer that contained 0.17% BSA. After exhaustive washing, the blots were incubated with a secondary goat anti-rabbit alkaline-phosphatase-conjugated IgG (Sigma) at a dilution 1 : 30 000 for 2 h. After four washings of 5 min each, the membranes were incubated with 66 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 111 μg/ml nitro blue tetrazolium (NBT) in Tris 0.19 M, $MgCl_2$ 1 mM until colour appeared. The image was scanned using the Gel Doc System with Molecular Analyst software (Bio-Rad). Replacing the antiserum with pre-immune serum was used as a negative control to rule out non-specific binding to the transferred proteins.

Glycoprotein detection

To detect glycoprotein spots, the gels were stained with Pro-Q Emerald 300 Glycoprotein Stain (Molecular Probes) as described by the manufacturer's protocol, and were scanned and analysed with PD-Quest™ 2-D analysis software (Bio-Rad).

Protein deglycosylation

Seminal plasma samples were subjected to deglycosylation using *N*-Glycosidase *F*-Deglycosylation kit (Roche, Penzberg, Germany) shown to be highly efficient with mainly all types of glycoproteins (Tarentino et al. 1985). Seminal plasma (50 μl) was boiled for 5 min in the presence of β-mercaptoethanol (1%, w/v) and SDS (0.2%, w/v) (Gatti et al. 2002). As the addition of a non-ionic detergent can be beneficial with native glycoproteins (Steube et al. 1985), we added 30 μl of 10% (w/v) CHAPS after cooling. The samples were then incubated overnight at 37°C with 3 U of *N*-glycosidase F (Roche).

Amino acid sequencing of the F6 map spots

Spots were excised from the gel and sent to the Proteomic Analysis Service (Parque Científico, Barcelona, Spain). Proteins were in-gel digested with trypsin (Sequencing grade modified, Promega, Madrid, Spain) in the automatic Investigator ProGest robot of Genomic Solutions. Briefly, excised gel spots were washed sequentially with ammonium bicarbonate buffer and acetonitrile. Proteins were reduced by treatment with 10 mM DTT, and alkylated with 100 mM iodine acetamide, for 30 min. After sequential washings with buffer and acetronitrile, proteins were digested with 0.27 nmol of trypsin overnight, at 37°C. Tryptic peptides were extracted from the gel matrix with 10% formic acid and acetonitrile. Extracts were pooled and dried in a vacuum centrifuge.

Peptides were analysed by either MALDI-TOF (Matrix-Associated Laser Desorption Ionization-Time of Flight)/TOF mass spectrometry (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA)

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or ESI-MS-MS (Q-TOF Global, Micromass-Waters, Milford, CA, USA). In the first case, the digests were redissolved in 5 μl of 0.1% trifluoroacetic acid in 50% acetonitrile. Typically, a 0.5 μl aliquot was mixed with the same volume of a solution composed of 5 mg/ml α-ciano-4-hydroxycinnamic acid (CHCA) (Aldrich, Madrid, Spain) in 0.1% trifluoroacetic acid and 50% acetonitrile. Major peaks were selected and further characterized by MS/MS analysis. Spectra were submitted for database searching in a generic MASCOT format (Perkins et al. 1999). Tryptic digests were also analysed by on-line liquid chromatography–nanoelectrospray ionization-tandem mass spectrometry (Cap-LC-nano-ESI-Q-TOF) (CapLC, Micromass-Waters). For this, digested and lyophilized samples were resuspended in 12 μl of 10% formic acid, and 4 μl was injected to chromatographic separation in reverse-phase capillary C_{18} column (75 μm of internal diameter and 15 cm length, PepMap column, LC Packings). The eluted peptides were ionized via coated nano-ES needles (PicoTip^{TM}, New Objective). A capillary voltage of 1800–2200 V was applied together with a cone voltage of 80 V. The collision in the CID (collision-induced dissociation) was 20–35 eV and argon was employed as collision gas.

The *de novo* sequencing allows a reconstruction of the peptide sequence using a protein database (Medzihradszky 2005). In addition, the introduction of modified amino acids into the reconstruction is usually less prohibitive than general database searches. The *de novo* algorithms used were PeqSeq de Micromass and Novo tool de Applied Biosystems. The assigned sequences were submitted to a search on the NCBI reference sequence database using the Blast algorithm (Altschul et al. 1997). For all searches, this database was filtered for mammalian proteomes, exclusively. The sequences submitted for comparison to this database corresponded to the longest single contiguous segment that was interpretable for each peptide.

Results

2D-PAGE reference map of ram seminal plasma proteins

A 2D-PAGE map of enhanced resolution was achieved by maximum solubilization of the sample by addition of TBP, and a polyacrylamide linear gradient gel with a narrow pH range (4–7). One representative ram SP gel stained with Sypro Ruby is presented in Fig. 1, where the main protein spots are designated by correlative numbering according to M_r. The image analysis software allowed detection of 195 protein spots, with isoelectric points (pIs) ranging from 4.5 to 6.6, and M_r from 11.7 to 90.4.

The most significant proteins in terms of their relative amount were spots 1, 4, 7, 8, 9 and 15, which had the highest relative intensity and accounted for medium values of 12.9%, 5.7%, 12.2%, 3.9%, 10.1% and 5.8%, respectively, of the quantified proteins in the gel. These proteins had low M_r (14.9, 15.1, 15.6, 15.7, 15.8 and 21.1) and acidic pI (5.8, 5.7, 5.2, 5.5, 5.6 and 6.6) respectively (Table 1).

Similar 2D-PAGE protein maps were found from SPs of different months, with excellent reproducibility as

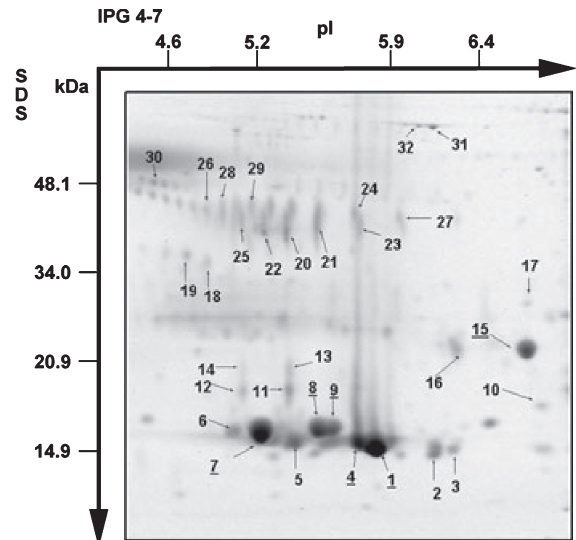

Fig. 1. Optimized 2-D electrophoresis of ram seminal plasma proteins. Isoelectric focusing (IEF) with linear immobilized pH gradient (IPG, pH 4–7) was carried out with 75 μg of SP proteins diluted in 125 μl of sample buffer containing 8 M urea, 2% [3-(3-(cholamydopropyl) dimethyl-ammonio)-1 propane sulphonate] (CHAPS), 40 mM dithiothreitol (DTT) and 3.2 mM tributyl phosphine (TBP). The 9–20% linear gradient SDS-PAGE gel stained with Sypro-ruby. Molecular weight markers indicated at the left and pH markers indicated on top of the gel are approximate. Underlined spots have the highest relative intensity

well as very low variability in the presence of protein spots.

Glycoprotein detection and protein deglycosylation

Despite improving the map resolution by using TBP and a linear gradient gel with a narrow pH, certain protein spots continue to show a tendency to aggregate, which could be due to hydrophobic domains confirmed by partition in a 2-phase system with Triton X114 (data not shown), which account for their tendency to aggregate. Therefore, we used a specific staining to find out which protein spots of ram SP are glycosylated. Fig. 2a shows that most significant proteins in ram SP are glycosylated, with the exception of certain minor spots (14, 17). The removing of N-linked oligosaccharides from proteins with N-glycosidase improved the gel resolution. As shown by Sypro-ruby staining (Fig. 2b), following protein deglycosylation, spots were focused and better separated. It is worth pointing out that four new spots were found as a consequence of partition of spots 20, 21, 22 and 23 that were split into two after treatment. The M_r of all resulted spots was lower than the original points.

2D-electrophoresis of fraction 6 of ram seminal plasma

Earlier, we have shown that one protein fraction separated from SP by exclusion chromatography on Sephacryl-100 HR (fraction 6 or F6) protects sperm against cold-shock membrane damage, and that the main components of this fraction, two protein bands of approximately 14 (RSVP14) and 20 (RSVPP20) kDa

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Table 1. Standard spot number (SSP), theoretical molecular weight (M_r) and isoelectric point (pI) of main protein spots identified in ram SP. Percentage of protein content of each spot (medium value ± SEM of four replicated samples).

SSP	M_r	pI	%	N-Glycosylated
1	14.9	5.8	12.9 ± 1.98	+
2	14.9	6.2	2.48 ± 0.40	+
3	14.9	6.3	1.24 ± 0.3	+
4	15.1	5.7	5.7 ± 0.52	+
5	15.3	5.4	2.72 ± 0.34	+
6	15.6	5.1	2.14 ± 0.48	+
7	15.6	5.2	12.2 ± 1.05	+
8	15.7	5.5	3.91 ± 1.7	+
9	15.8	5.6	10.1 ± 2.51	+
10	16.1	6.6	0.6 ± 0.04	+
11	18.2	5.4	1.89 ± 0.09	+
12	18.4	5.1	0.93 ± 0.06	+
13	20.0	5.4	0.2 ± 0.01	+
14	20.2	5.1	0.2 ± 0.00	–
15	21.1	6.6	5.8 ± 0.94	+
16	21.3	6.3	1.46 ± 0.24	+
17	26.9	6.6	0.22 ± 0.01	–
18	34.8	4.9	0.65 ± 0.11	+
19	35.9	4.8	0.72 ± 0.12	+
20	39.2	5.4	2.4 ± 0.41	+
21	39.2	5.5	2.18 ± 0.21	+
22	39.3	5.2	1.1 ± 0.16	+
23	39.5	5.8	0.74 ± 0.09	+
24	40.1	5.7	0.73 ± 0.12	+
25	40.4	5.1	0.93 ± 0.11	+
26	40.6	4.9	0.28 ± 0.06	+
27	40.8	6.0	0.64 ± 0.63	+
28	41.3	5.1	0.41 ± 0.08	+
29	42.1	5.1	0.21 ± 0.05	+
30	48.1	4.5	0.22 ± 0.03	+
31	75.7	6.1	0.54 ± 0.04	+
32	76.5	6.0	0.40 ± 0.03	+

(Fernández-Juan et al. 2006), are responsible for this protective effect (Barrios et al. 2005).

To characterize further the identified protein spots in ram SP, the whole F6 fraction was analysed by 2D-PAGE. The obtained 2D map showed six main protein spots, four (*a*, *b*, *c*, *d*) in the 14 kDa and two (*e*, *f*) in the 20 kDa region (Fig. 3). Estimation of pI accounted for the following values: 5.5, 5.2, 4.8 and 5.0 for spots *a*, *b*, *c* and *d*; 5.2 and 4.9 for spots *e* and *f* respectively. These protein spots matched to those identified in the SP map as 4, 5, 6 and 7 (*a*, *b*, *c* and *d*) and 13, 14 (*e*, *f*) respectively (Fig. 1).

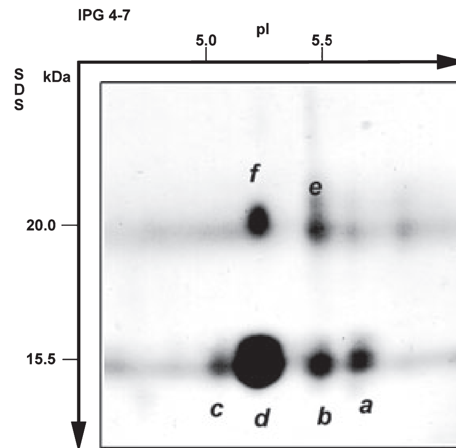

Fig. 3. Two-dimensional electrophoresis of the whole F6 of ram seminal plasma. Isoelectric focusing (IEF) with linear immobilized pH gradient (IPG, pH 4–7) was carried out with a mixture of 50 μg of F6 proteins diluted in 125 μl of sample buffer containing 8 M urea, 2% [3-(3-(cholamydopropyl) dimethyl-ammonio)-1 propane sulphonate] (CHAPS), 40 mM dithiothreitol (DTT), 3.2 mM of tributyl phosphine (TBP). The 9–20% linear gradient SDS-PAGE gel was stained with Coomassie R

Western-blot analysis of ram SP using anti-P14 and anti-P20 antibodies

Western-blot analyses using polyclonal antibodies raised against RSVP14 (anti-P14) or RSVP20 (anti-P20) bands, recovered from a non-denaturing polyacrylamide gel of the F6 of ram SP (Barrios et al. 2005), indicated that the anti-P14 antibody recognized four spots on the SP map, 4, 5, 6 and 7, that matched with spots *a*, *b*, *c* and *d* of F6 map (Fig. 4a). The anti-P20 antibody recognized spots 13 and 14 of SP map that corresponded to spots *e*, *f* of F6 map, although the spots identified by the anti-P14 antibody also showed reactivity with this antibody (Fig. 4b).

Amino acid sequencing

Spots of the F6 map were cut and sent to the Proteomic Analysis Service, Parque Científico, Barcelona, Spain.

MALDI-TOF/TOF, ESI-MS-MS, Cap-LC-nano-ESI-Q-TOF were performed without obtaining reliable data. Only the *de novo* sequencing of the peptide

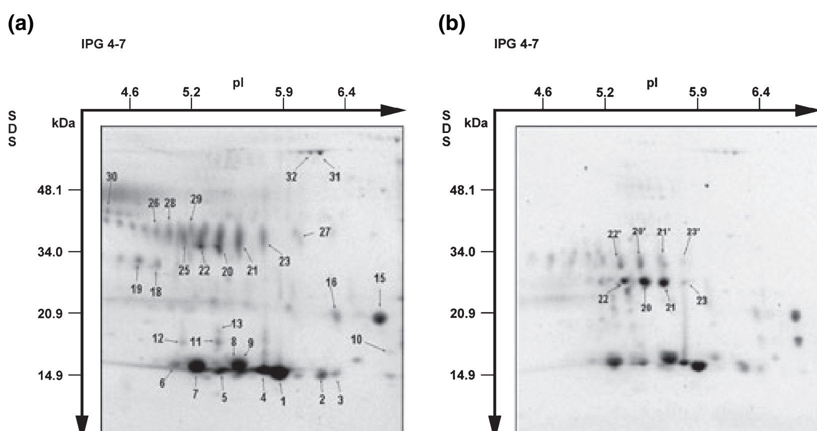

Fig. 2. Glycoprotein detection in ram seminal plasma. Two-dimensional 9–20% linear gradient SDS-PAGE gel stained with (a) Pro-Q Emerald 300 Glycoprotein Stain as described by the manufacturer's protocol; (b) the same sample subjected to deglycosylation using *N*-Glycosidase F-Deglycosylation kit, stained with Sypro-ruby. Molecular weight markers indicated at the left and pH markers indicated on top of the gel are approximate

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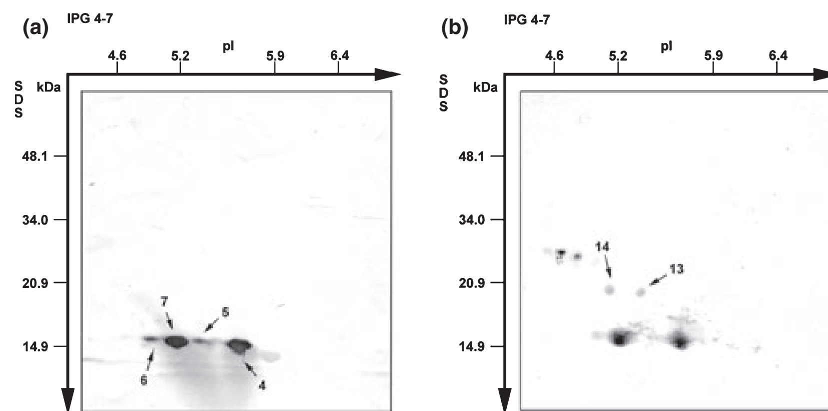

Fig. 4. Western-blot analyses using polyclonal antibodies raised against RSVP14 (anti-P14) or RSVP20 (anti-P20) bands, recovered from a non-denaturing gradient polyacrylamide gel of the F6 of ram SP. The gel, indicated in Fig. 1., was electrotransferred to PVDF, and subjected to immunodetection using (a) anti-P14 antibody and (b) anti-P20 antibody. Molecular weight markers indicated at the left and pH markers indicated on top of the gel are approximate

fragments obtained after trypsin digestion analysed by on-line liquid chromatography–tandem mass spectrometry accounted for trustworthy results. The sequences of all mammalian-related proteins were used to perform sequence comparisons with recent entries in databases. The deduced sequences were:

Spot 4 (*a* in the F6 map):	(FTL)DADYVGR
	(CGGS)DADYVGR
Spot 7 (*d* in the F6 map):	SLDADYVGR
	CSLDADYVGR
	SLSPNYDQDGAWK
Spot 13 (*e* in the F6 map):	(SL)DEDYVGR
	CHFPFLYR
	(EY)AWQYCDR
	(K)WCSLDEDYVGR
Spot 14 (*f* in the F6 map):	(GSG)DEDYVGR
	(FTL)DADYVGR
	(CGGS)DADYVGR

The amino acids in brackets might be in the opposite order. The proteins represented by spots 4 and 14 showed no similarities to any functionally characterized protein in the current Proteinbank databases. Spots 7 and 13 have significant similarities to bovine PDC-109 (Esch et al. 1983), also called BSP A1/A2, and to BSP A3 (Manjunath and Sairam 1987). Spot 7 also showed homology to HSP-1 (Calvete et al. 1995). Spots 5 and 6 were not detected at all.

Discussion

The present study shows the 2D-PAGE map of the ram SP obtained with a polyacrylamide linear gradient gel and a narrow pH range. The optimization of the 2D map was achieved by maximum solubilization of the sample after addition of TBP to the sample buffer, as previously reported (Rabilloud 1996; Gorg et al. 2004). Confirmation of this higher solubilization is evidenced by the absence of vertical streaking on the 2D map describing 195 protein spots. The high number of detected spots, compared with a recent work on ram SP using 12% acrylamide gels which differentiated 21 protein spots (Jobim et al. 2005), denotes the higher resolution of the gradient gel. In addition, the use of a narrow IPGs (4–7), not only increased the resolution but also gave more accurate information than wider IPGs (3–10) (Cardozo et al. 2006) because more spots were detected and better separated.

The results of this study showed that the 54.6% of the total protein identified in the gel corresponds to six main spots of acidic pI and low M_r. Most significant proteins in ram SP are glycosylated, and deglycosylation treatment improved protein spot separation. Thus, each spot 20, 21, 22 and 23 was split into two after treatment, all with lower M_r than the original points. These two resulted spots could correspond to different multimeric forms of the same protein. This could be due to changes in the aggregation state of polypeptide chains, as a consequence of the loss of glycosyl residues that would favour hydrophobic interactions. A reduction in the number of associated subunits, or a total loss of multimeric form, would account for a lower M_r.

Results of Western-blot confirmed that spots 4, 5, 6 and 7 of SP map correspond to RSVP14, based on the specific reaction of the anti-P14 antibody to those spots, and corroborate the findings found in the 2D map of F6. As previously reported (Barrios et al. 2005), we performed automated Edman degradation of the RSVP14 band recovered after electrophoresis using a non-denaturing polyacrylamide gel of the fraction 6 of ram SP. We only found one protein sequence although the process was carried out twice until the amino acid 35 was reached, and at least four times for the first 10 amino acids (Barrios et al. 2005). In that work, we also proved that RSVPP14 is phosphorylated at serine and threonine residues, but not glycosylated. However, the results of the present study using Pro-Q Emerald 300 Glycoprotein detection kit showed that the four protein spots identified at the 14 kDa region of the 2D-F6 map or with the anti-P14 antibody (4, 5, 6 and 7 of SP map) are glycosylated. This indicates the high sensitivity of this stain that only binds to carbohydrate groups at glycosylation sites. We might then suggest that these four spots correspond to the same protein with certain modification, as it could be due to a different degree of phosphorylation and/or glycosylation, which could account for differences in M_r and/or pI. Similarly, we found that the anti-P20 antibody raised against the RSVP20 band of ram SP (Barrios et al. 2005) reacted with protein spots 13 and 14 of SP map (spots *e* and *f* in the F6 map), although the spots identified with the anti-P14 antibody were also recognized. Therefore, we can

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conclude that, despite the fact that the anti-P20 antibody is not highly specific, spots 4, 5, 6 and 7 correspond to RSVP14 and might represent different forms of this protein, and spots 13 and 14 match with RSVP20. As we have proved in this study, and already reported (Barrios et al. 2005), RSVP20 is a glycosylated protein, and we could suggest that spots 13 and 14 might well correspond to the same protein with (spot 13) and without (spot 14) glycosylation.

The sequencing analyses of two spots that match with RSVP14 (4 and 7) evidenced that spot 7 has significant similarities to the BSP family (Manjunath and Sairam 1987). These results are consistent with those determined by N-terminal automatic sequencing of the RSVP14 fragment (recovered from a SDS-PAGE gel) which we previously reported (Barrios et al. 2005) that also showed a high similarity with several SP proteins of different species, particularly bovine PDC-109 (Esch et al. 1983), GSP-14/15 kDa (goat SP protein) related to the BSP family (Villemure et al. 2003) and the recently reported RSP-15/RSP-16 kDa of ram (Bergeron et al. 2005; Jobim et al. 2005). Interestingly, the sequence assigned to spot 4 showed no similarity with any reported protein. And spots 5 and 6, also found in F6 map and identified by the anti-P14 antibody like RSVP14, were not determined despite the numerous attempts made to find any sequence. If these two spots are only traces of the other protein spots identified by the antibodies, it could be assumed that the band called RSVP14 (Barrios et al. 2005; Fernández-Juan et al. 2006) is composed of two different proteins identified as spots 4 and 7 in the SP map. This would be consistent with previous results of 2D-electrophoresis with a wider pI range (3–10) in which we only detected two protein spots with the anti-P14 antibody (Cardozo et al. 2006).

Similarly, sequencing analyses of two protein spots identified as RSVP20 (13 and 14) showed important differences between them. Comparative sequence analysis (Expasy Molecular Server) revealed a relationship between spot 13 and several proteins, showing the highest homology with bovine PDC-109 and also with glucose-6-phosphate dehydrogenase. The sequence deduced for spot 14 did not appear to be homologous with any known protein in the current Proteinbank databases. This result also agrees with our previous study on N-terminal automatic sequencing of the RSVP20 fragment recovered from a SDS-PAGE gel (Barrios et al. 2005) that showed no significant similarity with any protein sequence collected in the databank, so protein spot 14 might probably be still a novel uncharacterized protein.

We have already proved (Barrios et al. 2005) that RSVP14 contains a part of the FN2 domain (Fibronectin Domain Type II) (Greube et al. 2001) as several SP proteins of other species, particularly bovine PDC-109 (Esch et al. 1983), GSP-14/15 kDa and RSP15 (Bergeron et al. 2005), all related to the BSP family. Due to the high homology between protein spots 7 and 13 and the BSP proteins, we could hypothesize that these two protein spots may take part in the protein structure surrounding the spermatozoa in a similar way to fibronectin, stabilizing membrane phospholipids and cytoskeleton. They could be involved in sperm capacitation and gamete interaction, stabilizing the sperm membrane in a first step (decapacitating factors), and in a later step, participating in the membrane modification during capacitation, as we have already postulated for RSVP14 and RSVP20 (Barrios et al. 2005). This could be the explanation for the protective effect of these two proteins against cold-shock injury on ram sperm membrane as we have evidenced their decapacitating role, and that both proteins are partially removed from the sperm surface at the beginning of the capacitation, although they are to some extent kept there after acrosome reaction (Barrios et al. 2005).

These results indicate that ovine SP contains important proteins for an accurate functioning of spermatozoa, and may contribute to the development of strategies for improving the fertilizing ability of ovine semen. On the basis of these results, it could be interesting to gain further knowledge of the functionality of specific proteins, and their relationship with sperm function.

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