Theriogenology 79 (2013) 1247–1261

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Membrane-associated proteins of ejaculated sperm from Morada Nova rams

M.F. van Tilburg [a], M.A.M. Rodrigues [a], R.A. Moreira [b], F.B. Moreno [b], A.C.O. Monteiro-Moreira [b], M.J.D. Cândido [a], A.A. Moura [a,*]

[a] Department of Animal Science, Federal University of Ceará, Fortaleza, Ceará, Brazil
[b] School of Pharmacy, University of Fortaleza, Fortaleza, Ceará, Brazil

ARTICLE INFO

Article history:
Received 17 November 2012
Received in revised form 20 February 2013
Accepted 16 March 2013

Keywords:
Ram
Sperm membrane
Seminal plasma
Proteomics
Binder of sperm proteins

ABSTRACT

The objective was to describe the profile of membrane proteins from sperm of tropically adapted Morada Nova rams (N = 5). Samples from protein-enriched fractions of ejaculated sperm (containing 400 μg of protein) were separated by two-dimensional electrophoresis and respective maps analyzed using PDQuest software (version 7.3.0; Bio-Rad). Proteins were identified using tandem mass spectrometry. Also, membrane proteins were incubated with antibodies against binder of sperm protein (BSP) 1 and bodhesin 2 (Bdh-2), components of vesicular gland secretion. For membrane proteins of ejaculated sperm, an average of 133 ± 4.6 spots were detected per gel, of which, 107 spots were consistently present on all gels. Sixty-eight spots and 37 proteins were identified using mass spectrometry, corresponding to 71.6% of the intensity of all spots detected. Three major spots identified as ram seminal vesicle protein (RSVP) 14 represented approximately 30% of the intensity of all spots. Two of the most intense spots in the gel reacted against anti-BSP1, at 14 kDa. In addition, four low molecular weight spots reacted with anti-Bdh-2 antibodies. Proteins RSVP and Bdh-2 belong to the BSP and spermadhesin families, respectively, and were previously reported as major components of ram seminal proteins. Additional proteins identified in the sperm membrane two-dimensional maps included alpha-2-heparan sulfate-glycoprotein, plasma glutamate carboxypeptidase, arylsulfatase A, cathelicidin, heat shock protein 70 kDa, angiotensin-converting enzyme, leucine aminopeptidase, and clusterin. Some proteins were present as multiple isoforms, such as tubulin (12), alpha-2-heparan sulfate-glycoprotein (5), ATP synthase (5), Bdh-2 (4) and RSVP14 (3). Based on gene ontology analysis, the most common biological processes associated with the membrane proteins were cellular processes (34%), response to stimulus (14%), and metabolic processes (11%). Binding (37%) and catalytic activity (32%) corresponded to the most frequent molecular functions for those proteins. In conclusion, we identified a diverse cohort of components of membrane proteins in ram sperm. Major proteins previously reported in seminal plasma, such as RSVP14 and Bdh-2, were also extracted from sperm membranes. Knowledge of sperm proteins is crucial for elucidating mechanisms underlying their association with sperm function.

1. Introduction

Sperm are formed in the testis as functionally immature and during epididymal transit and maturation, they undergo a series of changes including acquisition of motility, membrane remodeling, changes in intra-sperm pH, and protein tyrosine phosphorylation. Sperm maturation is

* Corresponding author. Tel.: +55 85 3366 9701; fax: +55 85 3366 9701.
E-mail address: amoura@ufc.br (A.A. Moura).

0093-691X © 2013 Elsevier Inc. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.theriogenology.2013.03.013

partially regulated by proteins present in the epididymal fluid [1]. However, sperm only attain their full fertilizing ability after ejaculation, when they come into contact with the secretions of the accessory sex glands and undergo a second series of biochemical and structural changes associated with sperm capacitation. Capacitation also requires cholesterol and/or phospholipid efflux from the sperm membrane, resulting in membrane reorganization, alterations in calcium uptake, and appearance of novel sperm surface antigens [2]. This lipid efflux is predominantly mediated by a family of proteins called binder of sperm proteins (BSPs), present in seminal plasma of several domestic mammals [3]. In rams, BSPs are known as ram seminal vesicle proteins (RSVPs) and expressed as RSVP14 (14 kDa) and RSVP22 (22 kDa). These proteins are mainly secreted by the vesicular glands and represent major components of semen, corresponding to approximately 20% of seminal proteins [4]. Seminal plasma proteins are also related to sperm function and fertility, including osteopontin, prostaglandin-D-synthase, spermadhesin Z13, and BSP 30 kDa [5,6], tissue inhibitor of metalloproteinase 2 [7], and angiotensin-converting enzyme (ACE) [8], among others.

Factors related to male sex contribute to a large proportion of infertility in several species and the nature of male subfertility is as complex as that of the female [9]. Several studies have pointed to the actions of diverse proteins present in semen of mammals, as related to the acquisition of sperm fertilizing capacity [10]. Recently, our research team has conducted comprehensive studies regarding reproductive development, age at puberty [11], spermatogenesis, and protein profiles of the seminal plasma from hairy rams [4]. Despite the large amount of information obtained during those studies regarding tropically adapted Brazilian rams, how sperm parameters relate to the expression of molecular components of the reproductive tract requires further investigtion. Therefore, the present study was conducted to identify the major profile of sperm membrane proteins from hairy rams.

2. Materials and methods

2.1. General procedures

Rams were managed in accordance with international guidelines for research involving animals. Semen samples were collected from five adult Morada Nova rams with normal reproductive status and seminal plasma was immediately separated from sperm using centrifugation. Protein extracts were obtained from the sperm membrane and subjected to two-dimensional (2-D) electrophoresis. All gels were stained with colloidal Coomassie blue and the resulting images, analyzed using PDQuest software (version 7.3.0; Bio-Rad, Rockville, MD, USA). Western blot analysis was also used to evaluate if BSP proteins and spermadhesins were present in protein extracts from sperm membranes. Spots of interest were identified using mass spectrometry.

2.2. Semen collection and extraction of membrane proteins

Semen samples were collected once from each animal, using an artificial vagina, and immediately placed in a water bath (37 °C). Aliquots were used for assessment of motion (scale from 1 to 5), the percentage of motile sperm, and sperm concentration (hemocytometer). An aliquot was also obtained for determination of the percentage of abnormal sperm, after staining cells with eosin and nigrosin and counting 200 cells per ejaculate [12]. The remaining semen was immediately centrifuged at 700 × *g* for 15 minutes at 4 °C to separate seminal plasma from sperm. Seminal plasma was discarded and sperm were washed three more times in cold PBS and stored at −20 °C.

Sperm were thawed at room temperature and washed three more times with PBS as mentioned previously herein. After the final wash, the sperm pellet was resuspended in 1.5 mL of PBS containing 1% (vol/vol) of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) containing 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (104 mM), aprotinin (80 μM), bestatin (4 mM), E-64 (1.4 mM), leupeptin (2 mM), and pepstatin A (1.5 mM). Membrane proteins were isolated as described by Bohring and Krause (1999) [13], with modifications. Sperm (500×10^6) from each sample were homogenized 20 times (3 seconds each) using a crusher-type device and washed three more times with PBS (5000 × *g*, 15 minutes, 4 °C) to remove intracellular proteins. The pellet containing membrane proteins was suspended in 2 mL of PBS containing 1% (vol/vol) Triton X-100 and incubated for 2 hours at 4 °C in mild agitation conditions. Samples were then sonicated for 30 minutes in iced water (4 °C) and centrifuged thereafter (5000 × *g*, 60 minutes, 4 °C). The supernatant was retained and proteins were precipitated with acetone by mixing 1 part of sample with 9 volumes of cold acetone (−20 °C) for 2 hours. Precipitated proteins were centrifuged (5000 × *g*, 60 minutes, 4 °C) and the pellet was air-dried overnight, followed by resuspension in a sample buffer (7 M urea, 2 M thiourea, 40 mM dithiothreitol [DTT], 2% free ampholytes [IPG buffer, pH 4-7 (GE LifeSciences)], 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate). Membrane proteins were diluted in water (1:50) and the total protein concentration was determined according to Bradford's method [14], using bovine serum albumin (Sigma-Aldrich) as standards. All Bradford assays were run in triplicates.

2.3. Two-dimensional electrophoresis and computerized analysis of the maps

The highly enriched ram sperm membrane proteins were separated using 2-D gel electrophoresis [15]. In summary, samples containing 400 μg of total protein were mixed with buffer (7 M urea, 2 M thiourea, 2% free ampholytes [IPG buffer, pH 4-7 (GE LifeSciences)], 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, and traces of bromophenol blue) sufficient to make 250 μL. Then, samples were incubated with 13-cm IPG strips (pH 4–7, linear; GE LifeSciences, Piscataway, NJ, USA), and allowed to rehydrate for 15 hours. Isoelectric focusing was carried out in an ETTAN IPGphor 3 (GE LifeSciences) isoelectric focusing system at 20 °C, according to the following program: 250 Volts (V) (750 Volts hours (Vh)), 500 V (500 Vh), 800 V (800 Vh), 1000 V (1000 Vh), 8000 V (1 hour, gradient), and 8000 V (32,000 Vh), with a total of 39,550 Vh.

After focusing, strips containing sperm membrane proteins were incubated in equilibration buffer I (6 M urea, 50 mM TRIS-HCl pH 8.8, 29.3% glycerol, 2% SDS, 1% DTT) and re-equilibrated for an additional 15 minutes in buffer II (similar to equilibration buffer I, but containing 2.5% iodoacetamide instead of DTT). After equilibration, strips were fixed with agarose (5% in SDS-PAGE running buffer) on the top of homogeneous SDS-polyacrylamide gels (12.5%) and run at 250 V, with 20 mA per gel (Hoefer SE 600; GE LifeSciences). Gels were stained in colloidal Coomassie blue [16], with modifications. Briefly, gels were washed three times (20 minutes each) in a solution containing phosphoric acid (2%) and ethanol (30%); another three washes in 2% phosphoric acid, and then put in a solution with phosphoric acid (2%), ethanol (18%), and ammonium sulfate (15%) added with 2 mL of a Coomassie Blue G-250 solution (2%) for 72 hours. Reagents used for electrophoresis were purchased from Bio-Rad, GE LifeSciences, and/or Sigma-Aldrich.

Two-dimensional gels were scanned at 300 dpi (ImageScanner II; GE LifeSciences), saved as tagged image file format (.tiff) files and analyzed using PDQuest software (version 7.3.0; Bio-Rad). According to a strategy previously reported [4,6,11,17], all 2-D maps were evaluated in a single-match set and a master gel was generated, based on a representative gel. In addition, spots consistently present in the remaining gels were added to the master, so that they could be matched to all samples. Proteins in key regions of the master gel were used as landmarks and final spot matches were organized by checking each spot in each gel with the respective pattern in the master. Based on PDQuest guidelines (version 7.3.0; Bio-Rad), protein quantities were given as parts per million of the total integrated optical density of spots in the gels.

2.4. Protein identification using electrospray ionisation quadrupole-time-of-flight mass spectrometry

For liquid chromatography-mlass spectrometry/mass spectrometry, samples were digested as described by Souza et al. [4]. Briefly, spots were excised from the gels and transferred to clean tubes, and washed three times with 400 μL of a solution containing acetronitrile (50%) and ammonium bicarbonate (50%; 25 mM at pH 8.0). Then the gel pieces were washed twice with 200 μL of acetonitrile for 5 minutes and air-dried at room temperature. Gel pieces were incubated with trypsin (166 ng per spot; Promega, cat #v511; Madison, WI, USA) for 20 hours at 37 °C. After the final incubation, peptides were extracted by three washings with 5% trifluoroacetic acid, 50% acetonitrile, in ammonium bicarbonate (50 mM). Supernatants were concentrated in micro tubes using a speed vacuum concentrator (Eppendorf, Hauppauge, NY, USA). Digested samples were injected using the nanoAcquity UPLC sample manager and the chromatographic separation was performed using a UPLC C18 column (75 μm × 10 cm) with a flow of 0.6 μL/min. The mass spectra were acquired in a Synapt G2 HDMS Acquity UPLC instrument (Waters Co., Milford, MA, USA) using data-dependent acquisition, in which the three top peaks were subjected to MS/MS. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The gradient conditions used were as follows: 0 minutes with 3% of B, increasing linearly to 30% B in 20 minutes, then it increased up to 70% B in 40 minutes where it remained until 50 minutes and in the next minute it was decreased to 3% of B. Data were processed using a Protein Lynx Global Server (Waters Co.) and subjected to database search using a Mascot search engine. The searches were made with the assumption that there was a maximum of one missed trypsin cleavage and that peptides were monoisotopic and using partially oxidized methionine residues, and completely carbamidomethylated cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to ± 0.1 Da, respectively, for MS/MS ion searching. However, candidate peptide identifications database were only accepted if the *m/z* values were observed within 0.1 Da (typically less than 0.05 Da) of the theoretical mass of the candidate identifications database, determined when manually reviewing Mascot search results.

2.5. Immunodetection of major seminal plasma proteins in sperm membrane extracts

Another sample of enriched-membrane proteins (300 μg) was separated by 2-D SDS-PAGE as described previously herein, but using 11-cm IPG strips (pH 4–7, linear; GE LifeSciences). Because the original 2-D gels of sperm membrane proteins did not have a significant number of spots in the basic range, we reduced the size of the 11-cm strips to 8 cm. This 8-cm strip was then used for electrofocusing and SDS-PAGE with the sperm membrane proteins. After running the gels, proteins were transferred to hydrophobic polyvinylidine difluoride membranes (8 × 9 cm; Hybond-P, GE LifeSciences) at 60 mA for 3 hours (TE 70 ECL Semi-Dry Transfer Unit; GE LifeSciences). Membranes were blocked overnight at 4 °C in 10 mL of PBS with 0.1% Tween 20 (PBS-T) containing skimmed milk powder (5% wt/vol), in mild agitation conditions, followed by incubation with anti-BSP1 primary antibodies (1:1000) or anti-bodhesin (Bdh) 2 (1:500) for 2 hours. Membranes were then washed three times in PBS-T and incubated with donkey anti-rabbit IgG coupled with alkaline phosphatase (Abcam) for another 2 hours, and washed more three times in PBS-T. The immunoreaction was visualized by exposing the membranes to a solution containing 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/mL), nitro blue tetrazolium (0.30 mg/mL), TRIS (100 mM), and $MgCl_2$ (5 mM), pH 9.5. The reaction was stopped by washing the membranes several times with ultrapure water. Controls consisted of incubation of another membrane with the primary or secondary antibodies alone (data not shown).

Antibodies against BSP1 were purified from rabbit antiserum and kindly provided by Dr. Puttaswamy Manjunath (Department of Medicine, University of Montréal, Canada). These antibodies cross-reacted with RSVP14, the ovine homologue of BSP1 [4]. Polyclonal antibodies against Bdh-2 were commercially developed (Rhea Biotech, Campinas, São Paulo, Brazil) and raised in rabbits against a peptide (SSNQPVSPFDIFYYERPSA) corresponding to the C-terminus sequence of Bdh-2 (gi: 121484235), as previously identified using MS/MS in ram seminal plasma samples [4].

2.6. Gene ontology analysis

Protein data obtained after Mascot search were analyzed using the software for researching annotations of proteins (STRAP), an open-source application [17]. Gene ontology terms for biological process, cellular localization, and molecular function were obtained from UniProtKB and EBI GOA databases.

3. Results

Rams used in this experiment ejaculated at least 1.0 mL of semen with an average score of 3.5 for wave motion, 80% motile sperm, and containing 1.5×10^9 sperm per mL, with a maximum of 5% of morphologically abnormal cells. Based on the analysis of gels conducted with the PDQuest software (version 7.3.0; Bio-Rad), an average of 133 ± 4.6 spots per gel of ejaculated sperm membrane proteins were detected. Among those, 107 spots were consistently present on all gels (Fig. 1). Sixty-eight spots and 37 proteins were identified using mass spectrometry (Fig. 1; Table 1), corresponding to 71.6% of intensity of all spots detected in the gels. Two of the most intense spots in the gel reacted against anti-BSP1, at 14 kDa, whereas four low molecular weight spots reacted with anti–Bdh-2 antibodies (Fig. 2). Additional proteins identified in the sperm membrane 2-D maps include alpha-2-heparan sulfate-glycoprotein (AHSG), plasma glutamate carboxypeptidase, arylsulfatase A, cathelicidin, heat shock protein 70 kDa, ACE, leucine aminopeptidase, and clusterin. Some proteins were present as multiple isoforms, such as tubulin (12), AHSG (5), ATP synthase (5), Bdh-2 (4), and RSVP14 (3), as shown (Fig. 1 and Table 1).

Based on gene ontology analysis, proteins were grouped according to biological process, molecular function, and cellular localization (Fig. 3). The most common biological processes associated with the proteins from our membrane protein-enriched samples were cellular processes (34%), response to stimulus (14%), and metabolic processes (11%). Binding (37%) and catalytic activity (32%) corresponded to the most frequent molecular functions for those proteins. Regarding their cellular localization, most of the proteins identified were associated with mitochondria (22%), cytoplasm (17%), and cytoskeleton (13%), and 17% of them were defined as extracellular and/or secreted proteins.

4. Discussion

The present study characterized the membrane protein profile of ejaculated sperm from adult and reproductively sound Morada Nova rams. These rams are typical of the semi-arid region of the Brazilian Northeast and are remarkably well adaptated to the tropical environment. Seminal plasma proteins are mostly derived from the cauda epididymis [16] and accessory sex glands [18], but they can also be shed from sperm membranes [19]. Certain seminal plasma proteins bind to sperm and many of them relate to several aspects of sperm function, such as capacitation [20], formation of the oviduct reservoir [21], and gamete interaction [22].

Using a 2-D PAGE-MS/MS approach, 68 spots were identified from the sperm membrane-enriched fraction, corresponding to 37 proteins. Some of these proteins have been reported in the ram seminal plasma [4], including RSVP14, Bdh-2, plasma glutamate carboxypeptidase, AHSG, actin, clusterin, heat shock protein 70 kDa, alpha enolase,

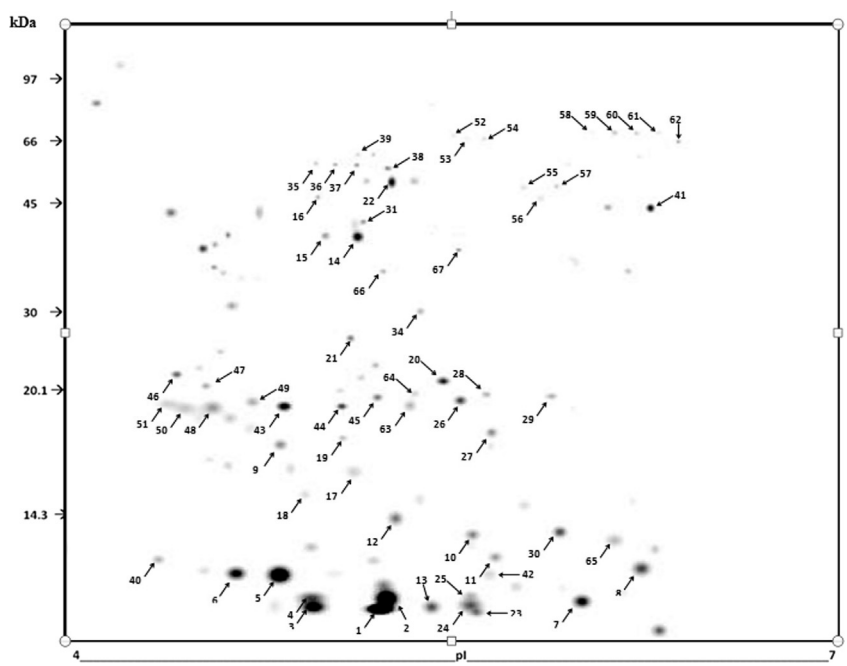

Fig. 1. Two-dimensional map of proteins of the sperm membrane-enriched fraction from Morada Nova rams. The map represents the master gel generated by PDQuest software (version 7.3.0; Bio-Rad), based on a match set with all gels used in the study. Proteins were stained with Coomassie blue and identified using mass spectrometry. Spot numbers refer to those shown in Table 1. pI, isoelectric point.

Table 1
Proteins of the sperm membrane-enriched fraction from Morada Nova rams identified by two-dimensional electrophoresis and mass spectrometry.

Protein	Experimental kDa/pI[a]	NCBI accession number	MS/MS protein score	Sequence covered (%)	Matched peptides[b]	Ion score	*m/z*	*z*
Ram seminal vesicles 14 kDa protein								
Spot 01	10.2/5.3	219521810	77	28	$^{(34)}$SSEESHEDEECVFPFTYK$^{(51)}$	37	740.9674	3
					$^{(80)}$FCSESDHANCVFPFIFGGK$^{(98)}$	42	1109.9824	2
Spot 02	10.6/5.3	219521810	104	30	$^{(32)}$DKSSEESHEDEECVFPFTYK$^{(51)}$	42	822.0129	3
					$^{(34)}$SSEESHEDEECVFPFTYK$^{(51)}$	38	1110.9570	2
					$^{(80)}$FCSESDHANCVFPFIFGGK$^{(98)}$	29	740.6502	3
Spot 05	11.4/5.0	219521810	91	30	$^{(32)}$DKSSEESHEDEECVFPFTYK$^{(51)}$	46	821.6810	3
					$^{(34)}$SSEESHEDEECVFPFTYK$^{(51)}$	21	740.6320	3
					$^{(80)}$FCSESDHANCVFPFIFGGK$^{(98)}$	29	1110.4761	2
Cytochrome C oxidase, chain E								
Spot 03	10.3/5.1	1942990	428	50	$^{(1)}$SHGSHETDEEFDAR$^{(14)}$	78	808.8346	2
					$^{(31)}$KGMNTLVGYDLVPEPK$^{(46)}$	61	888.9713	2
					$^{(32)}$GMNTLVGYDLVPEPK$^{(46)}$	71	824.9117	2
					$^{(47)}$IIDAALR$^{(53)}$	38	386.2409	2
					$^{(57)}$RLNDFASAVR$^{(66)}$	58	574.8142	2
					$^{(58)}$LNDFASAVR$^{(66)}$	73	496.7614	2
					$^{(67)}$ILEVVKDK$^{(74)}$	52	472.2967	2
Spot 04	10.7/5.1	1942990	269	49	$^{(1)}$SHGSHETDEEFDAR$^{(14)}$	34	539.5555	3
					$^{(32)}$GMNTLVGYDLVPEPK$^{(46)}$	70	824.9204	2
					$^{(47)}$IIDAALR$^{(53)}$	39	386.2383	2
					$^{(57)}$RLNDFASAVR$^{(66)}$	46	574.8138	2
					$^{(58)}$LNDFASAVR$^{(66)}$	40	496.7636	2
					$^{(67)}$ILEVVKDK$^{(74)}$	39	472.2955	2
Alpha-2-HS-glycoprotein								
Spot 06	11.3/4.9	57526674	43	5	$^{(313)}$HTFSGVASVESSSGEAFHVGK$^{(333)}$	43	707.3374	3
Spot 12	13.6/5.4	57526674	56	5	$^{(313)}$HTFSGVASVESSSGEAFHVGK$^{(333)}$	56	707.3368	3
Spot 15	39.6/5.1	57526674	40	5	$^{(313)}$HTFSGVASVESSSGEAFHVGK$^{(333)}$	40	707.3396	3
Spot 39	65.6/5.2	57526674	36	5	$^{(313)}$HTFSGVASVESSSGEAFHVGK$^{(333)}$	36	707.3389	3
Spot 48	20.3/4.9	57526674	43	5	$^{(313)}$HTFSGVASVESSSGEAFHVGK$^{(333)}$	43	707.3375	3
Tubulin, chain alpha 3								
Spot 07	68.3/6.2	84000299	103	3	$^{(113)}$EIVDLVLDR$^{(121)}$	62	536.2998	2
					$^{(157)}$LSVDYGKK$^{(164)}$	41	455.2574	2
Spot 10	13.0/5.8	84000299	85	3	$^{(113)}$EIVDLVLDR$^{(121)}$	56	536.3038	2
					$^{(157)}$LSVDYGKK$^{(164)}$	29	455.2568	2
Spot 27	18.7/5.8	84000299	254	10	$^{(113)}$EIVDLVLDR$^{(121)}$	41	536.3092	2
					$^{(157)}$LSVDYGK$^{(163)}$	19	391.2115	2
					$^{(157)}$LSVDYGKK$^{(164)}$	41	455.2589	2
					$^{(216)}$NLDIERPTYTNLNR$^{(229)}$	88	573.6318	3
					$^{(230)}$LIGQIVSSITASLR$^{(243)}$	69	729.4405	2
Spot 28	21.6/5.8	84000299	163	6	$^{(113)}$EIVDLVLDR$^{(121)}$	43	536.3091	2
					$^{(157)}$LSVDYGKK$^{(164)}$	35	455.2591	2
					$^{(216)}$NLDIERPTYTNLNR$^{(229)}$	88	573.6321	3
Spot 30	13.1/6.0	84000299	148	5	$^{(97)}$EDAANNYAR$^{(105)}$	60	512.7234	2
					$^{(113)}$EIVDLVLDR$^{(121)}$	50	536.3110	2
					$^{(157)}$LSVDYGKK$^{(164)}$	41	455.2598	2
Spot 64	21.6/5.5	84000299	141	6	$^{(113)}$EIVDLVLDR$^{(121)}$	43	536.3099	2
					$^{(157)}$LSVDYGKK$^{(164)}$	41	455.2598	2
					$^{(216)}$NLDIERPTYTNLNR$^{(229)}$	60	573.9613	3

(*continued on next page*)

Table 1 (*continued*)

Protein	Experimental kDa/pI[a]	NCBI accession number	MS/MS protein score	Sequence covered (%)	Matched peptides[b]	Ion score	*m/z*	*z*
Tubulin, chain beta 2B								
Spot 11	12.1/5.8	51491829	285	13	$^{(217)}$LTTPTYGDLNHLVSATMSGVTTCLR$^{(241)}$	55	908.7805	3
					$^{(242)}$FPGQLNADLR$^{(251)}$	78	565.8040	2
					$^{(242)}$FPGQLNADLRK$^{(252)}$	26	630.3428	2
					$^{(253)}$LAVNMVPFPR$^{(262)}$	64	580.3198	2
					$^{(263)}$LHFFMPGFAPLTSR$^{(276)}$	62	818.4056	2
Spot 19	18.6/5.0	51491829	150	6	$^{(155)}$IREEYPDR$^{(162)}$	19	539.2731	2
					$^{(163)}$ILNSFSVMPSPK$^{(174)}$	72	668.3550	2
					$^{(242)}$FPGQLNADLR$^{(251)}$	65	566.2979	2
Spot 45	21.3/5.3	51491829	332	16	$^{(104)}$GHYTEGAELVDSVLDVVR$^{(121)}$	24	653.6655	3
					$^{(155)}$IREEYPDR$^{(162)}$	30	539.2736	2
					$^{(163)}$ILNSFSVMPSPK$^{(174)}$	81	668.3547	2
					$^{(242)}$FPGQLNADLRK$^{(252)}$	63	565.8029	2
					$^{(253)}$LAVNMVPFPR$^{(262)}$	83	580.3211	2
					$^{(263)}$LHFFMPGFAPLTSR$^{(276)}$	51	546.2830	3
Spot 49	20.6/5.0	51491829	121	4	$^{(163)}$ILNSFSVMPSPK$^{(174)}$	79	668.3546	2
					$^{(242)}$FPGQLNADLRK$^{(252)}$	42	566.2999	2
Tubulin, chain beta 1								
Spot 20	22.8/5.7	221136900	349	9	$^{(155)}$IREEYPDR$^{(162)}$	32	539.2730	2
					$^{(163)}$ILNSFSVMPSPK$^{(174)}$	16	668.3559	2
					$^{(242)}$FPGQLNADLR$^{(251)}$	99	566.7908	2
					$^{(242)}$FPGQLNADLRK$^{(252)}$	54	630.3444	2
					$^{(252)}$KLAVNMVPFPR$^{(262)}$	79	644.3677	2
					$^{(253)}$LAVNMVPFPR$^{(262)}$	83	580.3211	2
Tubulin, chain beta								
Spot 17	16.1/4.9	2665736	327	41	$^{(47)}$YLTVAAVFR$^{(55)}$	50	520.3029	2
					$^{(58)}$MSMKEVDEQMLNVQNK$^{(73)}$	23	658.9491	3
					$^{(62)}$EVDEQMLNVQNK$^{(73)}$	44	732.3417	2
					$^{(88)}$TAVCDIPPR$^{(96)}$	33	514.7656	2
					$^{(100)}$MAVTFTGNSTAIQELFK$^{(116)}$	55	937.9706	2
					$^{(100)}$MAVTFTGNSTAIQELFKR$^{(117)}$	71	677.6788	3
					$^{(118)}$ISEQFTAMFR$^{(127)}$	68	623.7970	2
Acrosin-binding protein								
Spot 08	11.5/6.2	194666681	103	3	$^{(430)}$FYGLDLYGGLR$^{(440)}$	68	637.3334	2
					$^{(525)}$SEDLVLR$^{(531)}$	34	416.2362	2
Actin								
Spot 10	13.0/5.8	162606	71	11	$^{(108)}$DLTDYLMK$^{(115)}$	21	507.7466	2
					$^{(121)}$GYSFTTTAER$^{(130)}$	50	566.7694	2
Spot 51	20.3/4.8	110226713	221	12	$^{(19)}$AGFAGDDAPR$^{(28)}$	72	488.7310	2
					$^{(51)}$DSYVGDEAQSK$^{(61)}$	36	599.7683	2
					$^{(197)}$GYSFTTTAER$^{(206)}$	54	566.7687	2
					$^{(239)}$SYELPDGQVITIGNER$^{(254)}$	62	895.9544	2
ATP synthase, subunit beta, mitochondrial precursor								
Spot 13	10.2/5.6	28461221	75	3	$^{(189)}$VVDLLAPYAK$^{(198)}$	27	544.8236	2
					$^{(202)}$IGLFGGAGVGK$^{(212)}$	48	488.2869	2
Spot 16	47.6/5.1	28461221	481	20	$^{(110)}$TIAMDGTEGLVR$^{(121)}$	84	639.8249	2
					$^{(125)}$VLDSGAPIR$^{(133)}$	18	464.2660	2
					$^{(134)}$IPVGPETLGR$^{(143)}$	54	519.8023	2
					$^{(144)}$IMNVIGEPIDER$^{(155)}$	55	701.3583	2
					$^{(189)}$VVDLLAPYAK$^{(198)}$	45	544.8251	2
					$^{(202)}$IGLFGGAGVGK$^{(212)}$	28	488.2872	2

Spot 18	17.5/5.0	28461221	67	8	$^{(265)}$VALVYGQMNEPPGAR$^{(279)}$	31	809.9011	2
					$^{(282)}$VALTGLTVAEYFR$^{(294)}$	62	720.4024	2
					$^{(311)}$FTQAGSEVSALLGR$^{(324)}$	110	718.8782	2
					$^{(388)}$AIAELGIYPAVDPLDSTSR$^{(406)}$	31	994.5259	2
					$^{(463)}$FLSQPFQVAEVFTGHLGK$^{(480)}$	25	1004.0219	2
Spot 21	26.7/5.2	28461221	1036	36	$^{(481)}$LVPLKETIK$^{(489)}$	14	520.8433	2
					$^{(95)}$LVLEVAQHLGESTVR$^{(109)}$	73	826.4595	2
					$^{(110)}$TIAMDGTEGLVR$^{(121)}$	88	639.8245	2
					$^{(125)}$VLDSGAPIR$^{(133)}$	55	464.2685	2
					$^{(134)}$IPVGPETLGR$^{(143)}$	61	519.8012	2
					$^{(144)}$IMNVIGEPIDER$^{(155)}$	76	701.8518	2
					$^{(162)}$QFAAIHAEAPEFVEMSVEQEILVTGIK$^{(188)}$	100	1001.8372	3
					$^{(189)}$VVDLLAPYAK$^{(198)}$	36	544.8234	2
					$^{(202)}$IGLFGGAGVGK$^{(212)}$	45	488.2852	2
					$^{(213)}$TVLIMELINNVAK$^{(225)}$	67	738.4066	2
					$^{(226)}$AHGGYSVFAGVGER$^{(239)}$	69	703.8476	2
					$^{(240)}$TREGNDLYHEMIESGVINLK$^{(259)}$	67	778.7197	3
					$^{(242)}$EGNDLYHEMIESGVINLK$^{(259)}$	26	693.0014	3
					$^{(265)}$VALVYGQMNEPPGAR$^{(279)}$	71	810.3923	2
					$^{(282)}$VALTGLTVAEYFR$^{(294)}$	99	720.4036	2
					$^{(311)}$FTQAGSEVSALLGR$^{(324)}$	124	718.8772	2
Sperm acrosome-associated protein 5								
Spot 13	10.2/5.6	94966873	66	7	$^{(104)}$HILDDIMCAK$^{(113)}$	43	587.7860	2
					$^{(104)}$HILDDIMCAKK$^{(114)}$	23	454.2246	3
Spot 50	20.1/4.8	114053081	43	3	$^{(121)}$LACVHTSPENR$^{(131)}$	43	642.3154	2
Sodium channel protein type 9, subunit alpha								
Spot 14	39.4/5.2	160707935	46	1	$^{(585)}$GSLFVPHR$^{(592)}$	46	487.2716	2
Plasma glutamate carboxypeptidase precursor								
Spot 22	51.3/5.3	115495837	279	12	$^{(67)}$LALLVDTVGPR$^{(77)}$	94	577.3544	2
					$^{(87)}$AIEIMQQNLK$^{(96)}$	28	603.3104	2
					$^{(116)}$GEESAVMLEPR$^{(126)}$	66	617.2955	2
					$^{(197)}$VGALASLIR$^{(205)}$	59	450.2881	2
					$^{(262)}$SYPDADSFNTVAEITGSK$^{(279)}$	39	951.4480	2
Spot 31	41.2/5.2	115495837	268	10	$^{(67)}$LALLVDTVGPR$^{(77)}$	71	577.3555	2
					$^{(116)}$GEESAVMLEPR$^{(126)}$	74	617.2966	2
					$^{(197)}$VGALASLIR$^{(205)}$	62	450.2912	2
					$^{(262)}$SYPDADSFNTVAEITGSK$^{(279)}$	65	951.4484	2
Chaperonin containing TCP-1 subunit γ								
Spot 26	21.5/5.7	164448698	47	1	$^{(382)}$EILSEVER$^{(389)}$	47	488.2454	2
Spot 44	20.9/5.2	164448698	42	1	$^{(382)}$EILSEVER$^{(389)}$	42	488.2432	2
Glutathione S transferase								
Spot 29	21.1/6.0	114053087	266	18	$^{(23)}$MLLEFTDTSYEEK$^{(35)}$	77	811.3745	2
					$^{(23)}$MLLEFTDTSYEEKR$^{(36)}$	26	593.2841	3
					$^{(74)}$LTQSNAILR$^{(82)}$	72	508.7948	2
					$^{(131)}$YLEQLPGQLK$^{(140)}$	24	595.3311	2
					$^{(198)}$IATYMQSDR$^{(206)}$	78	551.2539	2
Spot 63	20.6/5.5	114053087	98	16	$^{(23)}$MLLEFTDTSYEEK$^{(35)}$	24	811.3712	2
					$^{(74)}$LTQSNAILR$^{(82)}$	77	508.7943	2
Pyruvate dehydrogenase E1, subunit beta, mitochondrial precursor								
Spot 34	29.7/5.5	164420789	279	19	$^{(53)}$VFLLGEEVAQYDGAYK$^{(68)}$	68	901.9541	2
					$^{(130)}$TYYMSGGLQSVPIVFR$^{(145)}$	90	917.9638	2

(*continued on next page*)

Table 1 (*continued*)

Protein	Experimental kDa/pI[a]	NCBI accession number	MS/MS protein score	Sequence covered (%)	Matched peptides[b]	Ion score	*m/z*	*z*
					$^{(259)}$EGIECEVINLR$^{(269)}$	48	666.3374	2
					$^{(309)}$IMEGPAFNFLDAPAVR$^{(324)}$	66	882.4515	2
					$^{(325)}$VTGADVPMPYAK$^{(336)}$	16	632.8199	2
Clusterin								
Spot 35	58.8/5.1	27806907	382	13	$^{(52)}$TQIEQTNEER$^{(61)}$	49	625.2853	2
					$^{(62)}$KLLLSSLEEAK$^{(72)}$	71	615.8738	2
					$^{(63)}$LLLSSLEEAK$^{(72)}$	57	551.8254	2
					$^{(153)}$IDSLMENDR$^{(161)}$	59	554.7562	2
					$^{(177)}$ASSIMDELFQDR$^{(188)}$	101	714.8232	2
					$^{(193)}$RPQDTQYYSPFSSFPR$^{(208)}$	59	659.6428	3
Arylsulfatase A								
Spot 35	58.8/5.1	115497982	141	7	$^{(85)}$MGLYPGVLEPSSR$^{(97)}$	58	711.3638	2
					$^{(303)}$GTTFEGGVR$^{(311)}$	41	462.2365	2
					$^{(371)}$HTLFFYSAYPDEVR$^{(384)}$	44	582.2829	3
Spot 36	59.1/5.2	115497982	141	8	$^{(59)}$FTDFYVPVSLCTPSR$^{(73)}$	13	895.4349	2
					$^{(85)}$MGLYPGVLEPSSR$^{(97)}$	80	711.3633	2
					$^{(371)}$HTLFFYSAYPDEVR$^{(384)}$	50	582.2824	3
Spot 37	58.8/5.2	115497982	153	7	$^{(85)}$MGLYPGVLEPSSR$^{(97)}$	66	711.3620	2
					$^{(303)}$GTTFEGGVR$^{(311)}$	35	462.2359	2
					$^{(371)}$HTLFFYSAYPDEVR$^{(384)}$	54	582.2830	3
Secernin 3								
Spot 38	57.9/5.3	344268354	53	1	$^{(371)}$TMLDDMR$^{(377)}$	53	457.2116	2
Alpha enolase								
Spot 41	44.5/6.2	296479148	272	16	$^{(16)}$GNPTVEVDLFTAK$^{(28)}$	54	695.8687	2
					$^{(33)}$AAVPSGASTGIYEALELR$^{(50)}$	73	902.9803	2
					$^{(72)}$TIAPALVSK$^{(80)}$	24	450.2839	2
					$^{(93)}$LMIEMDGTENK$^{(103)}$	30	656.7946	2
					$^{(127)}$GVPLYR$^{(132)}$	17	352.7100	2
					$^{(240)}$VVIGMDVAASEFYR$^{(253)}$	77	786.8933	2
Lysine-specific demethylase 3B-like								
Spot 43	20.1/5.1	358413126	51	1	$^{(279)}$ESIEGKDGR$^{(287)}$	41	495.7490	2
					$^{(1744)}$DAVGTLKAHESK$^{(1755)}$	16	445.9086	3
Homeobox protein Hox-A4								
Spot 46	23.2/4.8	2495319	49	9	$^{(53)}$DHKLPNTK$^{(60)}$	49	516.3051	2
Heat shock-related 70 kDa protein 2								
Spot 47	21.9/4.9	41386699	68	3	$^{(506)}$GCLSKDDIDR$^{(515)}$	41	561.7603	2
					$^{(525)}$SEDEANRDR$^{(533)}$	29	545.7706	2
Sodium-dependent neutral amino acid transporter B(0)AT2								
Spot 52	77.9/5.7	18203606	47	1	$^{(367)}$CIAENSEMIIK$^{(377)}$	47	633.3248	2
Angiotensin-converting enzyme								
Spot 53	76.6/5.7	297459052	103	1	$^{(231)}$FYGPELIDLR$^{(240)}$	103	611.8308	2
Stress-70 protein, mitochondrial								
Spot 54	75.4/5.8	77735995	47	3	$^{(349)}$AQFEGIVTDLIR$^{(360)}$	27	681.8698	2
					$^{(378)}$SDIGEVILVGGMTR$^{(391)}$	20	803.4510	2
Leucine aminopeptidase								
Spot 55	50.1/5.8	410689	170	9	$^{(209)}$FAEIVEENLK$^{(218)}$	22	596.3199	2
					$^{(224)}$TDVFIRPK$^{(231)}$	25	488.2873	2
					$^{(277)}$GITFDSGGISIK$^{(288)}$	27	597.8246	2

Dihydrolipoyllisine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial							
Spot 56	47.8/5.8	115497112	69	3	$^{(351)}$TIQVDNTDAEGR$^{(362)}$	63	660.3109	2
					$^{(426)}$MPLFEHYTR$^{(434)}$	37	403.8629	3
Cytosol aminopeptidase								
Spot 57	50.3/5.9	165905571	495	30	$^{(280)}$LGFMSAFVK$^{(288)}$	33	508.2696	2
					$^{(328)}$GLVVPVIR$^{(335)}$	36	426.7900	2
					$^{(69)}$EILNISGPPLK$^{(79)}$	20	591.3478	2
					$^{(85)}$TFYGLHEDFPSVVVVGLGK$^{(103)}$	30	688.6978	3
					$^{(201)}$RLMETPANEMTPTK$^{(214)}$	29	550.9323	3
					$^{(202)}$LMETPANEMTPTK$^{(214)}$	16	747.8464	2
					$^{(215)}$FAEIVEENLK$^{(224)}$	45	596.3190	2
					$^{(230)}$TDVFIRPK$^{(237)}$	23	488.2874	2
					$^{(268)}$GSPNASEPPLVFVGK$^{(282)}$	29	750.3933	2
					$^{(283)}$GITFDSGGISIK$^{(294)}$	75	597.8250	2
					$^{(295)}$AAANMDLMR$^{(303)}$	29	513.2263	2
					$^{(304)}$ADMGGAATICSAIVSAAK$^{(321)}$	24	855.9086	2
					$^{(418)}$LFEASIETGDR$^{(428)}$	22	619.3091	2
					$^{(432)}$MPLFEHYTR$^{(440)}$	77	605.2923	2
					$^{(458)}$SAGACTAAAFLK$^{(469)}$	58	584.7905	2
					$^{(506)}$TLIEFLFR$^{(513)}$	32	519.8054	2
Acetyl-coenzyme A synthase 2-like, mitochondrial								
Spot 58	75.8/6.1	27807441	118	5	$^{(108)}$SPESIALIWER$^{(118)}$	33	650.8500	2
					$^{(367)}$INQFYGAPTAYR$^{(378)}$	25	701.3474	2
					$^{(524)}$TEEGYYEITGR$^{(534)}$	63	659.3034	2
Spot 59	75.6/6.1	27807441	155	6	$^{(201)}$VVITFNQGLR$^{(210)}$	23	574.3327	2
					$^{(367)}$INQFYGAPTAYR$^{(378)}$	45	700.8543	2
					$^{(524)}$TEEGYYEITGR$^{(534)}$	52	659.3032	2
					$^{(535)}$MDDVINISGHR$^{(545)}$	42	424.8706	3
Spot 60	74.8/6.2	27807441	368	10	$^{(54)}$EPAAFWGPLAR$^{(64)}$	52	607.8230	2
					$^{(131)}$ELLETTCR$^{(138)}$	44	511.2552	2
					$^{(201)}$VVITFNQGLR$^{(210)}$	55	574.3298	2
					$^{(367)}$INQFYGAPTAYR$^{(378)}$	65	700.8542	2
					$^{(524)}$TEEGYYEITGR$^{(534)}$	55	659.3044	2
					$^{(535)}$MDDVINISGHR$^{(545)}$	84	636.8078	2
					$^{(609)}$YAVPDQVLVVK$^{(619)}$	22	616.3530	2
Spot 61	74.0/6.2	27807441	257	9	$^{(131)}$ELLETTCR$^{(138)}$	35	511.2531	2
					$^{(201)}$VVITFNQGLR$^{(210)}$	53	574.3289	2
					$^{(365)}$LKINQFYGAPTAYR$^{(378)}$	25	548.6203	3
					$^{(367)}$INQFYGAPTAYR$^{(378)}$	27	701.8412	2
					$^{(524)}$TEEGYYEITGR$^{(534)}$	49	659.3049	2
					$^{(535)}$MDDVINISGHR$^{(545)}$	64	636.8046	2
					$^{(609)}$YAVPDQVLVVK$^{(619)}$	15	616.3531	2
Glycerol-3-phosphate dehydrogenase, mitochondrial precursor								
Spot 62	67.7/7.0	154152051	707	24	$^{(58)}$EAQILTLK$^{(65)}$	22	458.2825	2
					$^{(101)}$DDFSSGTSSR$^{(110)}$	30	529.7262	2
					$^{(129)}$LDIEQYR$^{(135)}$	44	469.2388	2
					$^{(179)}$LYDLVAGSNCLK$^{(190)}$	16	677.3447	2
					$^{(228)}$MNLAIALTAAR$^{(238)}$	67	580.8303	2
					$^{(270)}$CKDVLTGEEFDVR$^{(282)}$	26	783.8658	2
					$^{(442)}$SMAEDTINAAVK$^{(453)}$	70	633.3102	2
					$^{(464)}$TVGLFLQGGK$^{(473)}$	36	510.3018	2
					$^{(519)}$WPIVGVR$^{(525)}$	22	413.7523	2

(*continued on next page*)

Table 1 (*continued*)

Protein	Experimental kDa/pI[a]	NCBI accession number	MS/MS protein score	Sequence covered (%)	Matched peptides[b]	Ion score	*m/z*	*z*
					$^{(526)}$LVSEFPYIEAEVK$^{(538)}$	27	762.4053	2
					$^{(543)}$EYACTAVDMISR$^{(554)}$	53	716.8126	2
					$^{(558)}$LAFLNVQAAEEALPR$^{(572)}$	86	821.4578	2
					$^{(573)}$IVELMGR$^{(579)}$	39	417.2328	2
					$^{(597)}$KFLYYEMGYK$^{(606)}$	37	679.3315	2
					$^{(598)}$KFLYYEMGYK$^{(606)}$	29	615.2844	2
					$^{(616)}$SEISLLPSDIDR$^{(627)}$	69	672.8569	2
					$^{(641)}$GFITIVDVQR$^{(650)}$	41	574.3297	2
					$^{(715)}$RVPIPVDR$^{(722)}$	35	476.2934	2
Peroxiredoxin 5								
Spot 65	12.7/6.2	339522297	245	26	$^{(63)}$VGDAIPSVEVFEK$^{(75)}$	38	695.3706	2
					$^{(81)}$VNLAELFK$^{(88)}$	16	467.2768	2
					$^{(108)}$THLPGFVEQAGALK$^{(121)}$	56	734.8983	2
					$^{(154)}$LLADPNGTFGK$^{(164)}$	46	566.8071	2
					$^{(185)}$RFSMVIEDGIVK$^{(196)}$	39	705.3813	2
					$^{(186)}$FSMVIEDGIVK$^{(196)}$	61	627.3295	2
Actin-related protein T2								
Spot 66	34.4/5.3	84000199	324	18	$^{(55)}$KYFVGEEALHR$^{(65)}$	60	450.2385	3
					$^{(56)}$YFVGEEALHR$^{(65)}$	26	610.8112	2
					$^{(78)}$GLITGWEDMEK$^{(88)}$	41	647.8068	2
					$^{(102)}$ANDQPVLMTEPSLNPR$^{(117)}$	57	899.9446	2
					$^{(188)}$DITEHLTR$^{(195)}$	39	492.7629	2
					$^{(196)}$LLLASGR$^{(202)}$	47	365.2377	2
					$^{(211)}$ALVDDIKEK$^{(219)}$	23	515.7961	2
					$^{(234)}$RPEEVLR$^{(240)}$	37	449.7629	2
Spot 67	37.7/5.7	84000199	481	23	$^{(23)}$AGLSGEIGPR$^{(32)}$	68	478.7649	2
					$^{(33)}$HVVSSVVGHPK$^{(43)}$	33	573.3292	2
					$^{(55)}$KYFVGEEALHR$^{(65)}$	62	450.2393	3
					$^{(56)}$YFVGEEALHR$^{(65)}$	79	610.8124	2
					$^{(78)}$GLITGWEDMEK$^{(88)}$	37	647.8085	2
					$^{(102)}$ANDQPVLMTEPSLNPR$^{(117)}$	88	899.9440	2
					$^{(188)}$DITEHLTR$^{(195)}$	46	492.7630	2
					$^{(196)}$LLLASGR$^{(202)}$	31	365.2383	2
					$^{(234)}$RPEEVLR$^{(240)}$	33	449.7620	2
					$^{(366)}$EFGTSVIQR$^{(374)}$	15	519.2697	2
ATP synthase, subunit delta, mitochondrial precursor								
Spot 40	11.7/4.7	28603800	123	13	$^{(137)}$AQSELLGAADEATR$^{(150)}$	97	716.3620	2
					$^{(157)}$IEANEALVK$^{(165)}$	31	494.2746	2
Cathelicidin-1 precursor								
Spot 42	11.0/5.9	27807341	307	34	$^{(41)}$AVDQLNEQSSEPNIYR$^{(56)}$	99	932.9379	2
					$^{(107)}$CEGTVTLDQVR$^{(117)}$	75	640.3002	2
					$^{(118)}$GNFDITCNNHQSIR$^{(131)}$	45	850.8445	2
					$^{(132)}$ITKQPWAPPQAAR$^{(144)}$	52	489.2660	3
					$^{(135)}$QPWAPPQAAR$^{(144)}$	47	562.2838	2
60 kDa Heat shock protein, mitochondrial								
Spot 09	18.7/5.1	262205483	555	18	$^{(222)}$GYISPYFINTSK$^{(233)}$	51	695.3588	2
					$^{(237)}$CEFQDAYVLLSEK$^{(249)}$	36	801.8757	2
					$^{(250)}$KISSVQSIVPALEIANAHR$^{(268)}$	46	678.7159	3
					$^{(251)}$ISSVQSIVPALEIANAHR$^{(268)}$	82	635.6894	3
					$^{(293)}$VGLQVVAVK$^{(301)}$	48	456.8002	2
					$^{(302)}$APGFGDNR$^{(309)}$	46	417.6935	2

					$^{(345)}$VGEVIVTKDDAMLLK$^{(359)}$	43	549.6388	3
					$^{(353)}$DDAMLLK$^{(359)}$	24	411.2107	2
					$^{(397)}$LSDGVAVLK$^{(405)}$	40	451.2726	2
					$^{(406)}$VGGTSDVEVNEK$^{(417)}$	61	617.3048	2
					$^{(406)}$VGGTSDVEVNEKK$^{(418)}$	38	454.5962	3
					$^{(421)}$VTDALNATR$^{(429)}$	66	480.7625	2
Bodhesin-2								
Spot 23	10.3/5.8	121484235	200	18	$^{(87)}$SSNQPVSPFDIFYYERPSA$^{(105)}$	102	975.0745	2
					$^{(87)}$SSNQPVSPFDIFYYERPSA$^{(105)}$	94	1102.0930	2
Spot 24	10.2/5.8	121484235	87	18	$^{(87)}$SSNQPVSPFDIFYYERPSA$^{(105)}$	87	1102.2568	2
Spot 25	10.5/5.8	121484235	34	18	$^{(87)}$SSNQPVSPFDIFYYERPSA$^{(105)}$	34	1102.5125	2

Spot numbers refer to those in Figure 1.
Abbreviations: HS, heparan sulfate; MS/MS, mass spectrometry; NCBI, National Center for Biotechnology Information; pI, isoelectric point; TCP, t-complex polypeptide.
[a] Experimental values were deduced from the two-dimensional map in the PDQuest software (version 7.3.0; Bio-Rad).
[b] Superscript numbers in parenthesis relate to the position of each peptide within the amino acid sequence of the complete protein, according to database search using Mascot search engine.

arylsulfatase A, leucine aminopeptidase, peroxiredoxin 5, cathelicidin 1, and ACE. Among these, arylsulfatase A, clusterin, AHSG, ACE, and leucine aminopeptidase have been detected in the cauda epididymal fluid of bulls [16] and rams [4] and likely bind to sperm during epididymal transit or when cells are stored in the cauda epididymis. In mice, arylsulfatase A activity involves glycoconjugate desulfation, binding to epididymal sperm [23] and participation in sperm–egg interaction [24]. Angiotensin-converting enzyme is a component of testicular sperm membrane and secreted into the cauda epididymal fluid [19]. The role of ACE in the male reproductive tract has not been completely elucidated, but it is known that this protein participates in the release of glycosylphosphatidylinositol-anchored proteins from sperm [8] during acrosome reaction and it is crucial for sperm–egg binding [25]. It is speculated that ACE is required for normal fertility considering that knockout male animals for germinal ACE are subfertile [26]. Furthermore, ACE regulates disintegrin and metalloprotease domain 3 translocation in epididymal sperm [27] and its shedding from sperm is mediated by metalloproteases [19], such as leucine aminopeptidase and plasma glutamate carboxypeptidase (also identified in the present study). Leucine aminopeptidase activity in the seminal plasma is associated with semen criteria [28] and is present in the acrosomal membrane [29]. Plasma glutamate carboxypeptidase is present as a transmembrane protein [30] and its secretion in body fluids is hormone-dependent [31]. Sperm maturation involves proteolysis [32] and leucine aminopeptidase and glutamate carboxypeptidase might participate in such a process. However, excessive proteolysis can be harmful to sperm and, therefore, it needs to be to be controlled. Alpha-2-HS-glycoprotein is a metalloproteinase inhibitor [33]; treatment of semen with anti-AHSG antibodies adversely affects sperm motility [34]. Binding of AHSG to cell membranes is mediated by annexins [35]; this protein likely optimizes zinc–metalloproteinase activity to ensure normal sperm function.

Clusterin and heat shock proteins were also identified in sperm membrane extracts. Clusterin secretion is induced by cell injury [36] and it has been previously identified as a component of sperm membranes in bulls [37] and rams [38]. Clusterin protects sperm against immune reactions [39] and binds defective cells in the epididymis [40]. In fact, the population of sperm with detectable clusterin is positively associated with the number of morphologically abnormal cells and inversely related to sperm motility in rams [38] and nonreturn rates of bulls [40]. Heat shock proteins (HSPs) are expressed in response to cell stress [41] and HSP 60- and 70-kDa families are known components of sperm in various species [42,43]. Whereas HSP 60 was primarily located in the sperm midpiece, associated with the mitochondria, HSP 70 appeared in the acrosome. Distribution of HSPs is modified by sperm capacitation and acrosome reaction [43] and these proteins are probably associated with protein folding. However, acrosomal HSP 70 has a specific role in gamete interaction and fertilization [44].

Other proteins described in the sperm membrane 2-D maps are RSVP14 and Bdh-2, which have also been reported as some of the major seminal plasma components of Santa Ines rams [4]. Ram seminal vesicle proteins belong to

the BSP family and there are two main types of RSVP in ram seminal plasma, one with 14 kDa (RSVP14) and another with 22 kDa (RSVP22). Both RSVPs are expressed as several isoforms and, based on kDa and isoelectric point values, these proteins appeared to be identical to the 15–16 kDa and 20–24 kDa proteins described in Suffolk rams [45]. In the present study, RSVP14 was detected in sperm membrane gels, but not RSVP22, presumably because RSVP22 does not interact or interacts very weakly with the sperm membrane after ejaculation, although it is clearly present in 2-D gels of seminal plasma from both Santa Ines [4] and Morada Nova rams (our unpublished results).

In bulls, BSPs bind to sperm during ejaculation and remain bound after sperm comes in contact with secretions of the oviduct [46], where they mediate formation of the sperm reservoir [21]. Addionally, BSPs regulate sperm capacitation by selectively removing cholesterol and phospholipids from the membrane [47]. However, if BSPs remain in contact with sperm for extended intervals, there is a continuous removal of phospholipids and cholesterol, which in turn becomes deleterious to the membrane structure and sperm motility [2]. As mentioned previously herein, certain proteins presently identified in the protein-enriched sperm membrane gels have also been detected in the seminal plasma. The procedure we used included several washes of ejaculated sperm and the fact that typical seminal plasma proteins, such as BSPs and Bdh-2, appeared in the protein extract of membranes emphasized the great affinity of such components for sperm. We previously determined (unpublished) that the longer sperm remain in contact with seminal plasma after ejaculation, the more intense are BSP and Bdh-2 spots in gels of protein-enriched membrane fractions. Also, when semen is collected and frozen and membrane proteins are extracted later, after thawing, BSP and Bdh-2 spots become more intense than when gels are made with proteins extracted from fresh semen. Similarly, a recent study [21] mentioned that the presence of BSP5 was more intense in frozen/thawed than in ejaculated bovine sperm. Binder of sperm protein 5 is a 30-kDa component of bull seminal plasma and belongs to the BSP family.

The identities of two spots (Nos. 24 and 25) from the sperm membrane protein gels were shown as Bdh-2, as determined by mass spectrometry. Not only anti–Bdh-2 reacted with those two spots, but also with spot No. 2. As commonly known, Western blots are more capable of detecting proteins expressed in very low abundance and this is probably the reason why that third spot No. 2 appeared in the Western blots. Anti-BSP1 reacted with spot Nos. 1 and 2, which had been also identified using mass spectrometry as RSVP14. Bodhesin-2 is a member of the spermadhesin superfamily and abundant in the seminal plasma of ungulates [48]. Spermadhesins originate from the accessory sex glands [49] and epididymis [16] and participate in the formation of the oviduct reservoir [50] and gamete interaction [22]. However, based on studies conducted in the boar, these proteins also work as decapacitating factors [51]. In dairy bulls, expression of spermadhesin Z13 in the accessory sex gland fluid was inversely related to fertility [6]. It is possible, thus, that this type of association results from the decapacitating properties of spermadhesin.

Membrane-enriched extractions of ram sperm also contained proteins from cell organelles, mainly from mitochondria, including cytochrome c oxidase, glycerol-3-phosphate dehydrogenase, peroxiredoxin 5, heat shock proteins 60 and 70 kDa, ATP synthase subunits, pyruvate dehydrogenase, alpha enolase, and 2-oxoglutarate dehydrogenase. Although heat shock proteins maintain the folding stage for mitochondrial proteins, as discussed already, most of those organelle proteins are involved in oxidation and/or reduction activities associated with energy metabolism [52]. Cytoskeletal proteins, such as actin and tubulin, were also present in the gels of protein-enriched

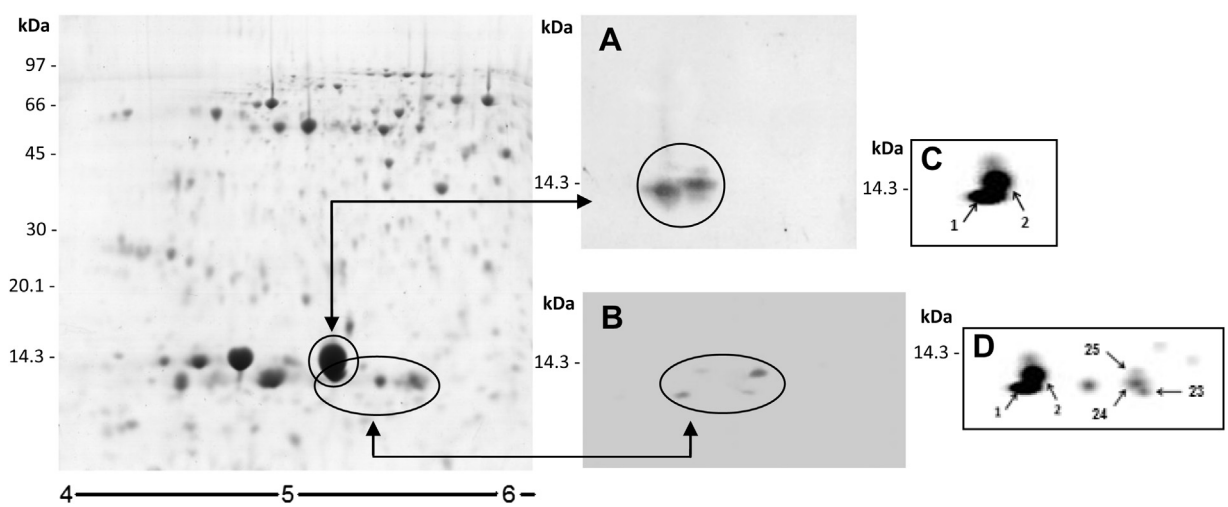

Fig. 2. Western blots of proteins from a sperm membrane-enriched fraction incubated with anti-binder of sperm protein 1 (A) and anti-bodhesin-2 (B). Proteins were separated using two-dimensional PAGE and transferred to hydrophobic polyvinylidine difluoride membranes. Spots and numbers in the small boxes (C) and (D) refer to those shown in Figure 1 and Table 1. Accordingly, spots 1 and 2 were identified using mass spectrometry as ram seminal vesicle protein 14 and spots 23, 24, and 25 as bodhesin-2.

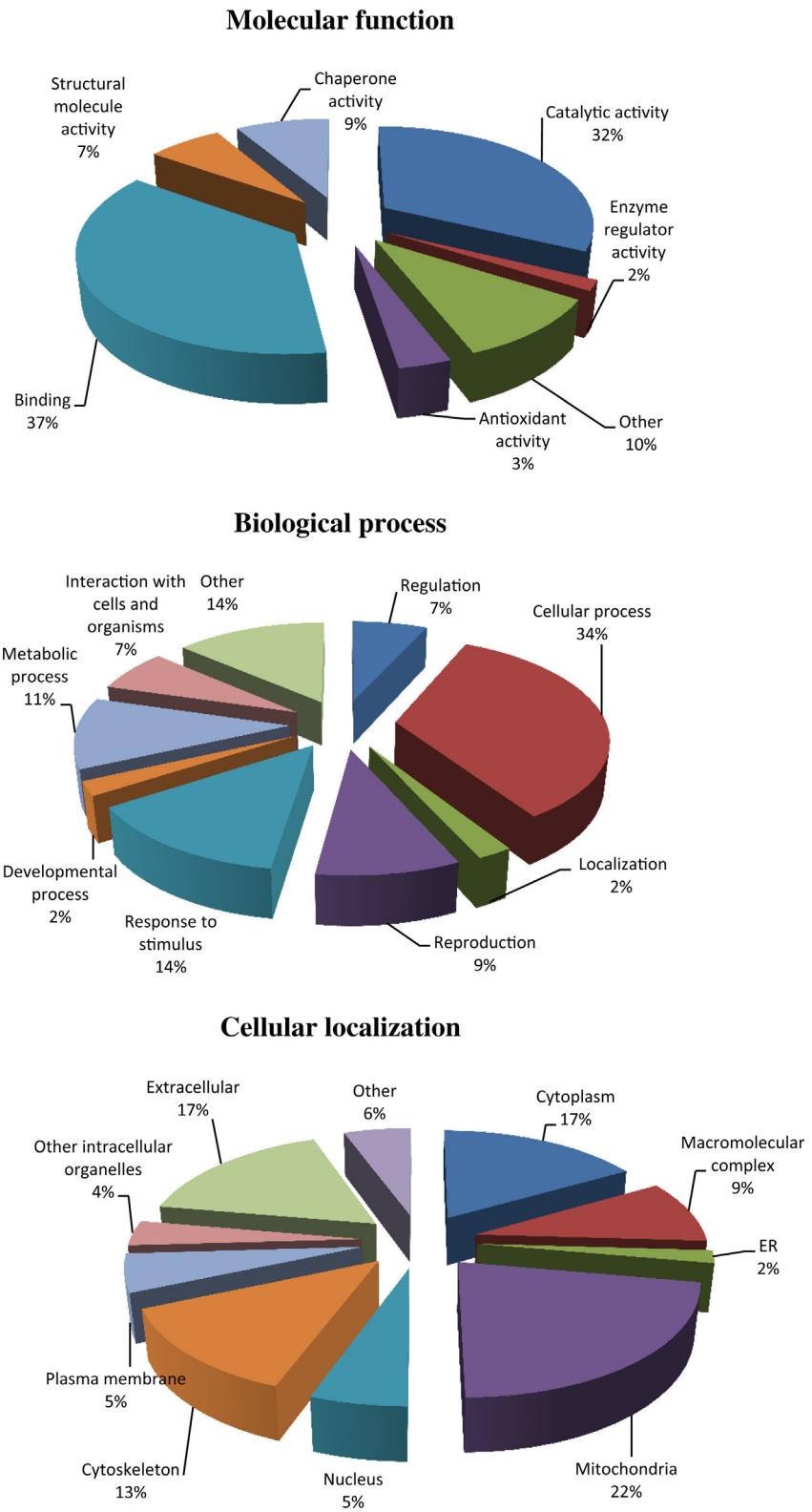

Fig. 3. Gene ontology annotations of the Morada Nova ram sperm membrane-enriched fraction proteome based on biological process, cellular localization, or molecular function. Protein data were analyzed using the software for researching annotations of proteins (STRAP [18]). Gene ontology terms were obtained from UniProtKB and EBI GOA databases. ER, endoplasmic reticulum.

sperm membrane. Our protocol not only separated membrane proteins but also reduced cytosolic protein fraction to salient membrane proteins. Furthermore, there is strong evidence for an association of actin with membrane structures [53] and actin is involved in intracellular changes associated with sperm capacitation and acrosome reaction [54]. Usually, tubulin exhibits multiple levels of penetration into the plasma membrane as the result of direct interactions with integral membrane proteins. Regulation of such interactions occur by attachment, via palmitoylation, to surface binding and to microtubules linked to membrane components. Integral membrane proteins are defined as structures that resist extraction by repeated washing with high salt, sodium carbonate (pH 10), or metal chelators. That definition might include proteins that do not deeply penetrate the plasma membrane but, instead, are membrane-bound by a lipid modification. Based on these criteria, a fraction of the total cellular tubulin is detected as integral to membranes [55]. Tubulins regulate intracellular reorganization in germ cells during spermiogenesis, an ATP-dependent process essential for normal fertility [56].

4.1. Conclusions

We identified a diverse cohort of components of membrane proteins present in ram sperm. Major proteins previously found in the seminal plasma, such as BSPs and spermadhesins, were also part of the proteins extracted from sperm membranes, indicating the strong interaction between seminal constituents and sperm. Knowledge of sperm proteins is a crucial step toward elucidating mechanisms underlying their association with sperm function.

Acknowledgments

Financial support for this research was provided by the Federal (CNPq, CAPES) and Ceará State (FUNCAP) Research Councils.

References

[1] Dacheux JL, Belleannée C, Jones R, Labas V, Belghazi M, Guyonnet B, et al. Mammalian epididymal proteome. Mol Cell Endocrinol 2009; 306:45–50.
[2] Moreau R, Frank PG, Perreault C, Marcel YL, Manjunath P. Seminal plasma choline phospholipid-binding proteins stimulate cellular cholesterol and phospholipid efflux. Biochim Biophys Acta 1999; 1438:38–46.
[3] Fan J, Lefebvre J, Manjunath P. Bovine seminal plasma proteins and their relatives: a new expanding superfamily in mammals. Gene 2006;375:63–74.
[4] Souza CE, Rêgo JP, Nogueira FC, Lobo CH, Oliveira JTA, Nogueira FS, et al. Proteomic analysis of the reproductive tract fluids from tropically-adapted Santa Ines rams. J Proteomics 2012;75:4436–56.
[5] Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in holstein bull seminal plasma. Biol Reprod 1993;49:1202–7.
[6] Moura AA, Koc H, Chapman DA, Killian GJ. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. J Androl 2006;27:201–11.
[7] McCauley TC, Zhang HM, Bellin ME, Ax RL. Identification of a heparin-binding protein in bovine seminal fluid as tissue inhibitor of metalloproteinases-2. Mol Reprod Dev 2011;58:336–41.
[8] Kondoh G, Tojo H, Nakatani Y, Komazawa N, Murata C, Yamagata K, et al. Angiotensin-converting enzyme is a GPI-anchored protein releasing factor crucial for fertilization. Nat Med 2005;11:160–6.
[9] Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim Reprod Sci 2000;60–61:663–77.
[10] Carballada R, Esponda P. Binding of seminal vesicle proteins to the plasma membrane of rat spermatozoa *in vivo* and *in vitro*. Int J Androl 1998;21:19–28.
[11] Souza CE, Araújo AA, Oliveira JT, Lima-Souza AC, Neiva JN, Moura AA. Reproductive development of Santa Inês rams during the first year of life: body and testis growth, testosterone concentrations, sperm parameters, age at puberty and seminal plasma proteins. Reprod Domest Anim 2010;45:644–53.
[12] Evans G, Maxwell WM. Handling and examination of semen. In: Salamon's artificial insemination of sheep and goats. Sydney: Butterworths; 1987. p. 93–106.
[13] Bohring C, Krause W. The characterization of human spermatozoa membrane proteins - surface antigens and immunological infertility. Electrophoresis 1999;20:971–6.
[14] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[15] Görg A, Postel W, Weser J, Günther S, Strehler IR, Hanash SM, et al. Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. Electrophoresis 1987;8:122–4.
[16] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, et al. Blue silver: a very sensitive colloidal coomassie g-250 staining for proteome analysis. Electrophoresis 2004;25:1327–33.
[17] Moura AA, Souza CE, Stanley BA, Chapman DA, Killian GJ. Proteomics of cauda epididymal fluid from mature holstein bulls. J Proteomics 2012;73:2006–20.
[18] Bathia VN, Perlman DH, Costello CE, Mccomb ME. Software tool for researching annotations of proteins: open-source protein annotation software with data visualization. Anal Chem 2009;81: 9819–23.
[19] Moura AA, Chapman DA, Koc H, Killian GJ. A comprehensive proteomic analysis of the accessory sex gland fluid from mature holstein bulls. Anim Reprod Sci 2007;98:169–88.
[20] Thimon V, Métayer S, Belghazi M, Dacheux F, Dacheux JL, Gatti JL. Shedding of the germinal angiotensin I-converting enzyme (gACE) involves a serine protease and is activated by epididymal fluid. Biol Reprod 2005;73:881–90.
[21] Manjunath P, Thérien I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 2002;53:109–19.
[22] Hung PH, Suarez SS. Alterations to the bull sperm surface proteins that bind sperm to oviductal epithelium. Biol Reprod 2012;87:88.
[23] Topfer-Petersen E, Ekhlasi-Hundrieser M, Tsolova M. Glycobiology of fertilization in the pig. Int J Dev Biol 2008;52:717–36.
[24] Weerachatyanukul W, Xu H, Anupriwan A, Carmona E, Wade M, Hermo L, et al. Acquisition of arylsulfatase a onto the mouse sperm surface during epididymal transit. Biol Reprod 2003;69:1183–92.
[25] Tantibhedhyangkul J, Weerachatyanukul W, Carmona E, Xu H, Anupriwan A, Michaud D, et al. Role of sperm surface arylsulfatase a in mouse sperm-zona pellucida binding. Biol Reprod 2002;67: 212–9.
[26] Watanabe H, Kondoh G. Mouse sperm undergo GPI-anchored protein release associated with lipid raft reorganization and acrosome reaction to acquire fertility. J Cell Sci 2011;124:2573–81.
[27] Yamaguchi R, Yamagata K, Ikawa M, Moss SB, Okabe M. Aberrant distribution of adam3 in sperm from both angiotensin-converting enzyme (ACE)- and calmegin (Clgn)-deficient mice. Biol Reprod 2006;75:760–6.
[28] Heder G, Siems WE, Nehring H, Muller K, Hilse H, Jentzsch KD. Occurrence and potential importance of selected peptidases in bull ejaculates. Andrologia 1989;21:247–55.
[29] Togo T, Morisawa M. GPI-anchored aminopeptidase is involved in the acrosome reaction in sperm of the mussel *Mytilusedulis*. Mol Reprod Dev 2004;67:465–71.
[30] Castelletti D, Alfalah M, Heine M, Hein Z, Schmitte R, Fracasso G, et al. Different glycoforms of prostate-specific membrane antigen are intracellularly transported through their association with distinct detergent-resistant membranes. Biochem J 2008;409:149–57.
[31] Suban D, Zajc T, Renko M, Turk B, Turk V, Dolenc I. Cathepsin C and plasma glutamate carboxypeptidase secreted from Fischer rat thyroid cells liberate thyroxin from the N-terminus of thyroglobulin. Biochimie 2012;94:719–26.
[32] Gatti JL, Castella S, Dacheux F, Ecroyd H, Métayer S, Thimon V, et al. Post-testicular sperm environment and fertility. Anim Reprod Sci 2004;82–83:321–39.

[33] Hedrich J, Lottaz D, Meyer K, Yiallouros I, Jahnen-Dechent W, Stocker W, et al. Fetuin-A and cystatin C are endogenous inhibitors of human meprin metalloproteases. Biochemistry 2010;49:8599–607.
[34] Pillai S, Rust PF, Howard L. Effects of antibodies to transferrin and alpha 2-HS glycoprotein on *in vitro* sperm motion: implications in infertility associated with endometriosis. Am J Reprod Immunol 1998;39:235–42.
[35] Chen NX, O'Neill KD, Chen X, Duan D, Wang E, Sturek MS, et al. Fetuin-A uptake in bovine vascular smooth muscle cells is calcium dependent and mediated by annexins. Am J Physiol Renal Physiol 2007;292:599–606.
[36] Bailey R, Griswold MD. Clusterin in the male reproductive system: localization and possible function. Molec Cell Endocrinol 1999;151: 17–23.
[37] Ibrahim NM, Troedsson MH, Foster DN, Loseth KJ, Farris JA, Blaschuk O, et al. Reproductive tract secretions and bull spermatozoa contain different clusterin isoforms that cluster cells and inhibit complement-induced cytolysis. J Androl 1999;20:230–40.
[38] Ibrahim NM, Romano JE, Troedsson MH, Crabo BG. Effect of scrotal insulation on clusterin-positive cells in ram semen and their relationship to semen quality. J Androl 2001;22:863–77.
[39] Meri S, Jarva H. Complement regulatory proteins. Nature Encyclopedia of Life Sciences 2001;1:1–8.
[40] Ibrahim NM, Gilbert GR, Loseth KJ, Crabo BG. Correlation between clusterin-positive spermatozoa determined by flow cytometry in bull semen and fertility. J Androl 2000;21:887–94.
[41] Mortensen CJ, Choi YH, Ing NH, Kraemer DC, Vogelsang MM, Hinrichs K. Heat shock protein 70 gene expression in equine blastocysts after exposure of oocytes to high temperatures in vitro or in vivo after exercise of donor mares. Theriogenology 2010;74:374–83.
[42] Miller D, Brough S, Al-Harbi O. Characterization and cellular distribution of human spermatozoal heat shock proteins. Hum Reprod 1992;7:637–45.
[43] Volpe S, Galeati G, Bernardini C, Tamanini C, Mari G, Zambelli D, et al. Comparative immunolocalization of heat shock proteins (HSP)-60, -70, -90 in boar, stallion, dog and cat spermatozoa. Reprod Domest Anim 2008;43:385–92.
[44] Spinaci M, Volpe S, Bernardini C, De Ambrogi M, Tamanini C, Seren E, et al. Immunolocalization of heat shock protein 70 (HSP 70) in boar spermatozoa and its role during fertilization. Mol Reprod Dev 2005;72:534–41.
[45] Bergeron A, Villemure M, Lazure C, Manjunath P. Isolation and characterization of the major proteins of ram seminal plasma. Mol Reprod Dev 2005;71:461–70.
[46] Souza CEA, Moura AA, Monaco E, Killian GJ. Binding patterns of bovine seminal plasma proteins a1/a2, 30 kda and osteopontin on ejaculated sperm before and after incubation with isthmic and ampullary oviductal fluid. Anim Reprod Sci 2008;105:72–89.
[47] Thérien I, Bleau G, Manjunath P. Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. Biol Reprod 1995;52:1372–9.
[48] Topfer-Petersen E, Romero A, Varela PF, Ekhlasi-Hundrieser M, Dostàlovà Z, Sanz L, et al. Spermadhesins: a new protein family. Facts, hypotheses and perspectives. Andrologia 1998;30: 217–24.
[49] Song CY, Gao B, Wu W, Wang XY, Chen GH, Mao J. Spatial and temporal expression of spermadhesin genes in reproductive tracts of male and female pigs and ejaculated sperm. Theriogenology 2010;73:551–9.
[50] Talevi R, Gualtieri R. Molecules involved in sperm-oviduct adhesion and release. Theriogenology 2010;73:796–801.
[51] Vadnais ML, Roberts KP. Seminal plasma proteins inhibit *in vitro*-and cooling-induced capacitation in boar spermatozoa. Reprod Fertil Dev 2010;22:893–900.
[52] Jiang Y, Wang X. Comparative mitochondrial proteomics: perspective in human disease. J Hematol Oncol 2012;5:11.
[53] Peterson RN, Bozzola JJ, Hunt WP, Darabi A. Characterization of membrane-associated actin in boar spermatozoa. J Exp Zool 1990; 253:202–14.
[54] De Las Heras MA, Valcarcel A, Pérez LJ, Moses DF. Actin localization in ram spermatozoa: effect of freezing/thawing, capacitation and calcium ionophore-induced acrosomal exocytosis. Tissue Cell 1997; 29:47–53.
[55] Wolff J. Plasma membrane tubulin. Biochim Biophys Acta 2009; 1788:1415–33.
[56] Kierszenbaum AL, Rivkin E, Tres LL. Cytoskeletal track selection during cargo transport in spermatids is relevant to male fertility. Spermatogenesis 2011;1:221–30.