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A comprehensive proteomic analysis of the accessory sex gland fluid from mature Holstein bulls

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Abstract

The expression of proteins in accessory sex gland fluid (AGF) of proven, high use mature Holstein bulls was evaluated. Thirty-seven bulls with documented fertility based on their non-return rates were studied. AGF was obtained by artificial vagina after bulls were surgically equipped with cannulae in the vasa deferentia. Samples of AGF were evaluated by two-dimensional SDS-PAGE, gels stained with Coomassie blue and polypeptide maps analyzed by PDQuest software. A master gel generated by the software representing the best pattern of spots in the AGF polypeptide maps was used as a reference for protein identification. Proteins were identified by Western blots and capillary liquid chromatography–nanoelectrospray ionization tandem-mass spectrometry (CapLC–MS/MS). The product ion spectra were processed using Protein Lynx Global Server 2.1 prior to database search with both PLGS and MASCOT (Matrix Science) software. The entire NCBI database was considered for mass fingerprint matching. An average of 52 ± 5 spots was detected in the AGF 2D gels, which corresponded to proteins potentially involved in capacitation (bovine seminal plasma protein—BSP-A1/A2 and A3, BSP 30 kDa, albumin); sperm membrane protection, prevention of oxidative stress, complement-mediated sperm destruction and anti-microbial activity (albumin, clusterin, acidic seminal fluid protein—aSFP, 5′-nucleotidase—5′-NT, phospholipase A_2—PLA_2); acrosome reaction and sperm-oocyte interaction (PLA_2, osteopontin); interaction with the extracellular matrix (tissue inhibitor of metalloproteinase 2, clusterin) and sperm motility (aSFP, spermadhesin Z13, 5′-NT). The 20 spots distinguished in all gels were matched to proteins associated with these functions. Proteins identified by tandem mass spectrometry as ecto-ADP-ribosyltransferase 5 and nucleobindin, never described before in the accessory sex gland secretions, were also detected. In summary, we identified a diverse range of components in the

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accessory sex gland fluid of a select group of Holstein bulls with documented fertility. Known characteristics of these proteins suggest that they play important roles in sperm physiology after ejaculation.

Keywords: Accessory sex gland; Bull; Proteomics

1. Introduction

It is commonly accepted that sperm acquires the capacity to fertilize oocytes in vivo while in the epididymis (Bedford, 1966; Amann and Griel, 1974), but there is also evidence that secretions of the accessory sex glands (AG) influence sperm physiology and fertilization. Mouse embryos sired by males without the AG have impaired development (Ying et al., 1998; Chow et al., 2003) and, from another prospective, secretions of the accessory sex glands are able to enhance the fertilizing capacity of bovine sperm collected from the cauda epididymis (Henault et al., 1995). Functions of sperm that may be affected by seminal plasma proteins include capacitation and the acrosome reaction (Einspanier et al., 1993; Yanagimachi, 1994; Aumuller et al., 1997; Visconti and Kopf, 1998; Atlas-White et al., 2000; Manjunath and Thérien, 2002), as well as motility (Elzanaty et al., 2002; Curi et al., 2003), DNA integrity (Chen et al., 2002) and interaction with the oocyte (Riffo and Párraga, 1997; Yuan et al., 2003).

Although many seminal plasma proteins have been studied in the bull and some of them are secreted by the accessory sex glands, a broad description of the components synthesized exclusively by the AG "in vivo" remains to be established. We previously utilized a technique to obtain fluid from the accessory sex glands and cauda epididymis by cannulation of the vasa deferentia of bulls (Henault et al., 1995). This approach enables the collection of a composite of AG secretions "mixed" by a living animal and that had not been in contact with either germ cells or other components from the testis and epididymis. Using this model, we recently identified four proteins of the accessory sex gland fluid (bovine seminal plasma protein—BSP 30 kDa, osteopontin, phospholipase A_2 and spermadhesin Z13) that are significantly related to fertility of Holstein bulls (Moura et al., 2006). However, we also believe that a comprehensive approach to identify proteins of accessory sex gland fluid, beyond these four associated with fertility scores, is crucial to understanding the potential mechanisms by which epididymal sperm function is altered after ejaculation. Thus, we presently report a proteomic analysis of the accessory sex gland fluid from mature Holstein bulls and discuss how known attributes of the proteins may influence the male gamete and fertilization.

2. Materials and methods

2.1. Sample collection

Accessory sex gland fluid (AGF) was collected by artificial vagina from 37 mature Holstein bulls that had been surgically altered by inserting indwelling catheters in the vasa deferentia (Henault et al., 1995). This procedure was approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University. Fluid from the accessory sex glands was centrifuged at $2400 \times g$ for 15 min immediately after collection and stored in liquid nitrogen until use. All bulls were assigned a fertility score expressed as the percentage point deviation (PD) of its non-return rate (NRR) from the average NRR of all bulls in a given artificial insemination center,

according to the method described by Killian et al. (1993). Frozen semen from each bull had been used to inseminate numerous cows (from 269 to 77,321 cows/bull) through sales of semen by artificial insemination centers in the Northeastern United States. The average NRR of bulls used in this study was 66.8 ± 1%. The percentage point deviation of their NRR was 1.7 ± 0.9, ranging from +7.7% to −18.1% (Moura et al., 2006).

2.2. Eletroctrophoresis

Samples of AGF were removed from liquid nitrogen, thawed at room temperature and centrifuged at 10,000 × *g* (60 min at 5 °C). The supernatant was then assayed for protein content (Lowry et al., 1951) using BSA as standards and aliquots, frozen at −80 °C. When used for electrophoresis, samples were thawed at room temperature and subjected to 2D electrophoresis (Killian et al., 1993). Isoelectric focusing was carried out in tube gels (Bio Rad, Rockville Centre, NY, USA) containing a mixture of ampholytes with pH from 3 to 7 (0.4 ml) and 3 to 10 (0.1 ml; Serva, Heidelberg, Germany). Samples of AGF containing 500 μg of protein were brought to a volume of 100 μl using a solution of β-mercaptoethanol, urea and the same ampholytes used in the gels. Gels were then subjected to 200 V for 15 min, 300 V for 30 min, 400 V for 30 min, 375 V for 16–18 h and 800 V during 1 h. Following focusing, gels were removed from the tubes and proteins were separated using gels with a linear gradient of acrylamide (10–17.5%). Standards from 66 to 14 kDa were also used (Sigma Chemical Co., St. Louis, USA). Gels were stained with Coomassie brilliant blue R-250, destained in a solution of methanol, acetic acid and deionized distilled H_2O and scanned using a GS-670 imaging densitometer (Bio Rad, Rockville Centre, NY). Images were analyzed using PDQuest 7.3.0 (Bio Rad, Rockville Centre, NY). A master gel was generated by the software, which represented the best pattern of spots in the AGF polypeptide maps. A few additional spots, consistently present in some gels, were also added to the master so that they could be matched to all samples. Proteins in key regions of the gels were used as landmarks and final matching of spots was achieved after several rounds of extensive comparisons and by checking each spot in each gel with the respective pattern in the master. Protein quantities were given as PPM of the total integrated optical density of the spots, according to PDQuest.

2.3. Protein identification

Proteins separated by 2D SDS-PAGE and selected by PDQuest were subjected to in-gel trypsin digestion (Koc et al., 2001). Excised gel pieces were washed three times with 100 μl of ammonium bicarbonate (25 mM) and dehydrated with 100 μl of acetonitrile (50%), and dried in a speed vacuum. They were then incubated overnight at 37 °C with trypsin (12.5 ng/μl in 25 mM ammonium bicarbonate). Peptides were then extracted twice with 25 μl of formic acid (5%) for 20 min. The extracts were dried in a speed vacuum and resuspended in 10 μl of 5% acetonitrile with formic acid (0.1%). Tryptic digests were analyzed by capillary liquid chromatography–nanoelectrospray ionization-tandem mass spectrometry (CapLC–MS/MS). A Micromass Q-Tof API US mass spectrometer coupled with a Waters CapLC HPLC unit was used for the analysis. As previously described (Abbas et al., 2005), the proteolytic digests (1–5 μl) were injected into solvent A (acetonitrile/water/formic acid, 5/95/0.1) supplied by the auxiliary pump of the capillary HPLC unit and trapped in a Waters Symmetry 300^{TM} column (C-18, 5 μm film; 0.3 mm × 5 mm) for on-line desalting and pre-concentration. After washing for 3 min with solvent A at 20 μl/min, trapped peptides were then back-flushed with the gradient solvent flow on to the analytical column, a Dionex PepMap fused silica capillary column (C-18 5 μm, 0.075 mm × 150 mm), using a 10-port

switching valve. The analytical column was run with a gradient (5–42% solvent B; acetonitrile/water/formic acid; 95/5/0.2; in 44 min. The mass spectrometry was calibrated using Glu-Fib product ion fragments as needed to maintain mass accuracy within10 ppm. The Q-Tof mass spectrometer was operated to acquire MS/MS of tryptic peptides in data-dependent acquisition mode for precursor ion selection using charge-state recognition and intensity threshold as selection criteria using MassLynx 4.0 SP1. In order to carry out the tandem mass spectrometric data acquisition, a survey scan (2 s) over the *m/z* of 400–1500 was performed. From each survey scan, up to four most intense precursor ions based on the selection criteria were selected for tandem mass spectrometry to obtain the production spectra resulting from collision-induced dissociation in the presence of argon. The product ion spectra (6–8 s) collected were processed using Protein Lynx Global Server 2.1 and were converted to peak list text files for database searching. In order to identify the proteins, MS/MS ion searches were performed on the processed spectra against a locally maintained copy of the NCBI NR database using MASCOT Daemon and search engine (Matrix Science, Inc., Boston, MA). The searches were made with the assumption that there was 1 maximum missed trypsin cleavage and that peptides were monoisotropic and oxidized at methionine residues (variable modifications) and carbamidomethylated at cysteine residues (fixed modification). Peptide mass tolerance and fragment mass tolerance were initially set to 1.2 and 0.6 Da, respectively, for MS/MS ion searching. However peptide mass values were ensured to be within 0.1 Da (typically less than 0.05 Da) when manually reviewing MASCOT search results.

2.4. Western blots

Proteins separated by 2D SDS-PAGE were transferred to a nitrocellulose membrane (Westran^{TM} S, Schleicher and Schuell Bioscience, Inc., Keene, NH, USA) using a Multiphor II Nova Blot (Pharmacia, USA) at 208 A for 1 h. Blots were then blocked overnight at 4 °C with heat inactivated goat serum (10 ml), PBS-Tween 20 (200 ml PBS with 0.5% Tween 20) and BSA (3%, w/v), followed by incubation with the primary antibody (Cancel et al., 1997) in 100 ml of blocking buffer for 2 h at room temperature. Membranes were then washed three times in PBS-Tween at room temperature (20 min each), incubated (1:7500) with goat anti-rabbit IgG HRP (Sigma Co., St. Loius, MO, USA) in 100 ml of blocking buffer for 1 h and again washed three times again in PBS-Tween (20 min each). Antibody reaction on the membranes was visualized with chemiluminescence (ECL^{TM} Western blotting detection reagents, Amersham Biosciences, USA) and exposition to a Kodak film (X: OMAT LS, Kodak Co., Rochester, NY, USA). Bovine seminal proteins BSP A1/A2 and A3 (Manjunath and Thérien, 2002) were detected using antibodies isolated from respective rabbit antiserum, kindly provided by Dr. P. Manjunath (Department of Medicine, University of Montreal, Canada). A column with protein-A Sepharose matrice (Sepharose CL-4B; Sigma Co., St. Loius, MO, USA) was washed initially with 50 nM PBS containing 0.15 M NaCl (pH 7.4) and adsorbed proteins were eluted with glycine–HCl (pH 2.5). Fractions with absorbance at 280 nm were pooled, which contained basically immunoglobins, including the anti-BSP antibodies, and immediately adjusted to pH 7.4 with 0.1N NaOH. The antibody solution was then aliquoted and stored in −20 °C until use. The blocking buffer dilutions used for the anti-BSP A1/A2, BSP A3 and BSP 30 kDa immunoblots were 1:10,000; 1:3,000 and 1:9,000, respectively.

3. Results

On average, 52 ± 5 spots were detected by PDQuest in the group forming the match set of 37 AFG gels, with a maximum of 61 and minimum of 40 spots per gel. Based

on the master image (Fig. 1A), which included all the proteins in the reference gel (Fig. 1B) and spots from other members of the match set, 36 spots were identified by CapLC–MS/MS and corresponded to 13 different proteins (Table 1). Intensity of spots related to these proteins accounted for 98.6% of the intensity of all spots shown on the master gel (Fig. 1A).

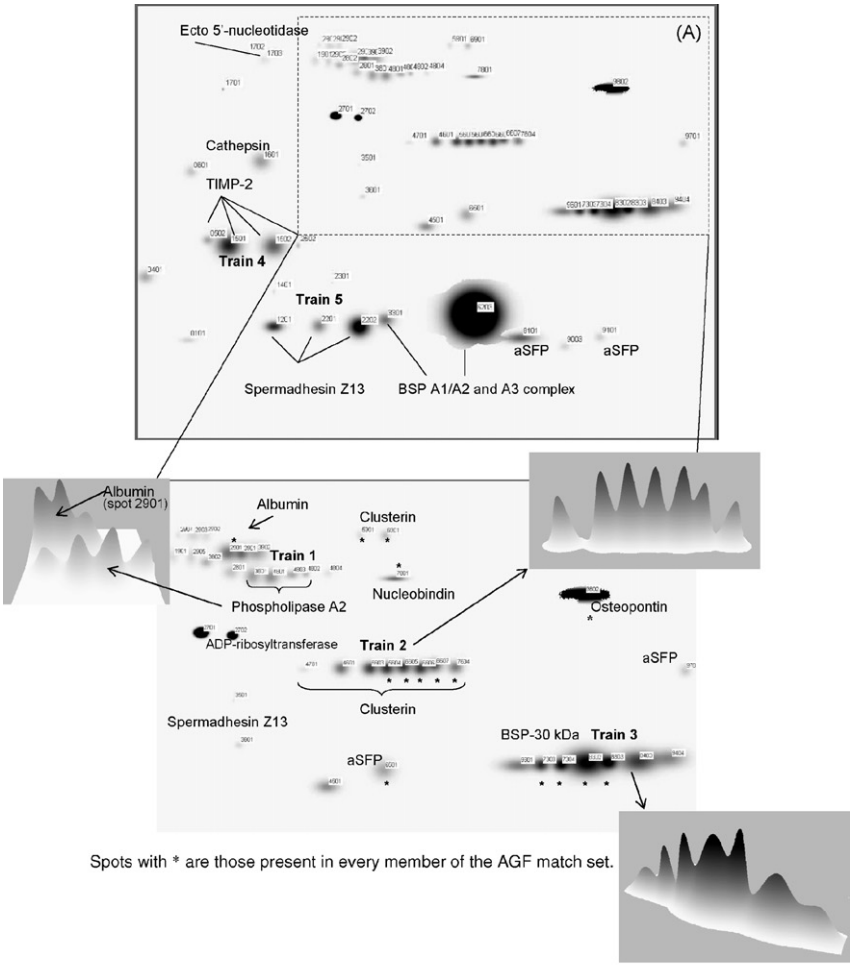
The large cluster of spots at 14 kDa (p*I* 5.8) was analyzed as a single unit by PDQuest (spot 6203; Fig. 1A and B). Tandem mass spectrometry analysis of trypic peptides from that spot matched to bovine seminal protein Pdc-109 and antibodies against BSP-A1/A2 and BSP-A3 reacted with these respective proteins in that region (Fig. 2A and B). Collectively, the intensity of the BSP A1/A2 and A3 complex (spot 6203) represented 86.5% of all proteins stained by Coomassie blue in the AGF maps. Another BSP protein was identified as BSP 30 kDa (spots 9601–9404, Train 3; Fig. 1A and B), but representing only 2.8% of the intensity of all spots in the master gel. Only five spots of Train 3 (spots 7603 through 8403), from a total of seven, were matched to BSP 30 kDa (Table 1) when analyzed by tandem mass spectrometry. Three of those spots had low protein scores but Western blots confirmed that an anti-BSP 30 kDa antibody reacted with the entire region of the AGF gel delimited as Train 3 (Fig. 3).

Twenty spots appeared consistently in every single member of the match set constructed by PDQuest (Fig. 1A). Based on their optical densities, this group of spots accounted for 96.6% of all spots detected in the AGF master gel and had an average coefficient of variation (CV) of 87.6 ± 9.1%. These spots matched to isoforms of clusterin at 70 kDa (spots 5901 and 6901) and at 40 kDa (Train 2), albumin (spot 2901), nucleobindin (spot 7801), osteopontin (spot 9802), a 28 kDa acidic seminal fluid protein—aSFP (spot 6501), tissue inhibitor of metalloproteinase-2 (Train 4), 14 kDa spermadhesin Z13 (Train 5), isoforms of BSP 30 kDa (Train 3) and the complex formed by BSP A1/A2 and A3 at 14 kDa (Fig. 1A). Within this group, proteins with the lowest CV were BSP A1/A2 and A3 complex (35.8%), tissue inhibitor of metalloproteinase 2 (41.9%), low molecular weight spermadhesin Z13 isoforms (46.5%) and nucleobindin (52.1%). The highest CVs were associated with the expression of osteopontin (105.8%), 70 kDa clusterin isoforms (163.6%) and the acidic seminal fluid protein at 28 kDa (141%).

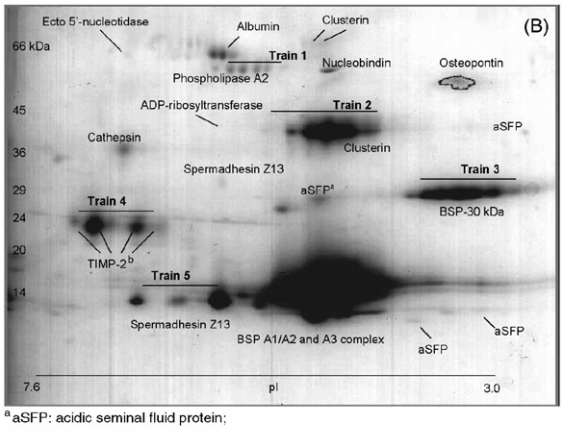
Although components of Train 1 (58 kDa; p*I* 6.0–6.2) were not present in every gel, every member of the match set had at least one of those spots, which matched to type VII phospholipase A_2 (Fig. 1; Table 1). Proteins that were not present in all 37 maps of the accessory sex gland fluid included low molecular weigh isoforms of aSFP (spots 8101 and 9101), cathepsin L (spot 1601), ecto 5′-nucleotidase (spot 1703) and ecto-ADP-ribosyltransferase 5—ART 5 (spot 2702). Those isoforms of aSFP were detected in 32 gels and cathepsin L, in 28 samples. Nucleotidase and ART-5 had measurable expression only in 9 and 7 bulls, respectively. Both ART-5 and nucleobindin are two new proteins found in the accessory sex gland fluid and Fig. 4A and B shows the product ion spectrum of trypic peptides originated from them.

4. Discussion

We report a comprehensive protein profile of accessory sex gland fluid of Holstein sires. These bulls represent a population of tested, mature sires that have been extensively used for artificial insemination of a large number of cows. Although such bulls were selected primarily for their ability to transmit desirable genetic characteristics associated with type and milk yield, they have also been screened for fertility merit. Sires with poor semen quality that do not meet the standards



Spots with * are those present in every member of the AGF match set.



[a] aSFP: acidic seminal fluid protein;

[b] TMP-2: tissue inhibitor of metalloproteinase 2

[c] Spots 6203-1 and 6203-2 were analyzed separately by CapLC-MS/MS and matched to Pdc 109.

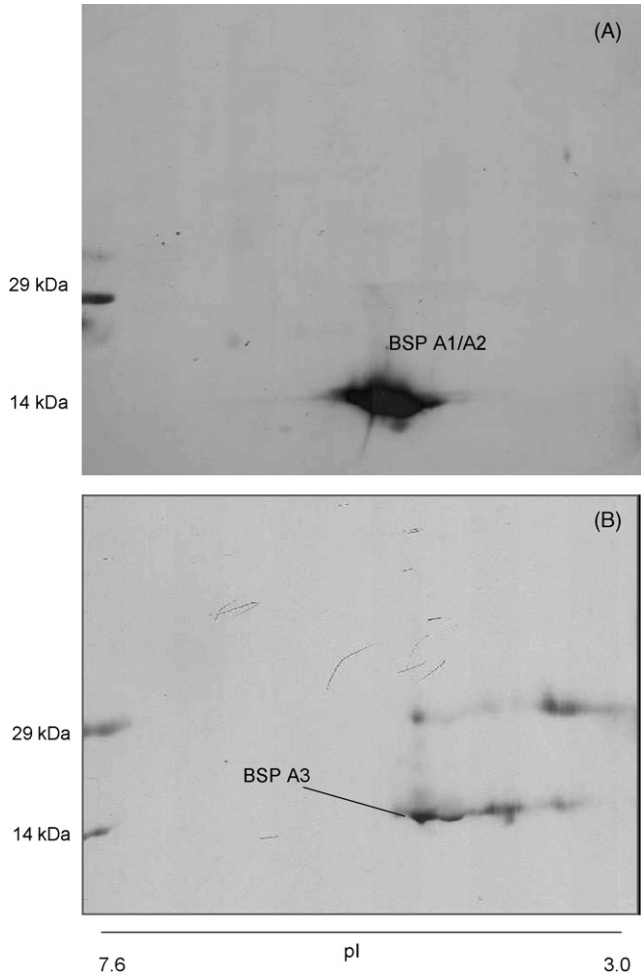


Fig. 2. Western blots of accessory sex gland fluid proteins incubated with anti-BSP A1/A2 (A) and A3 (B). Proteins were separated by 2D SDS-PAGE and transferred to nitrocellulose membranes.

of the artificial insemination industry are culled prior to entering commercial production of semen. A distinct advantage of using these bulls in a proteomic analysis is that their characteristics are transmitted to a large population of offspring which enables an evaluation of the composite of AG proteins in a defined population of bulls of known fertility. To our knowledge, this is the first study to report a proteomic analysis of the accessory gland fluid from bulls with these characteristics.

Fig. 1. Protein profile of the bull accessory sex gland fluid obtained by 2D SDS-PAGE and tandem mass spectrometry–CapLC–MS/MS. (A) The master gel constructed by PDQuest (spots with asterisk (*) are those present in every member of the AGF match set) and (B) the reference map (from bull #2) from which the master was mostly generated (letter a—aSFP: acidic seminal fluid protein; letter b—TMP-2: tissue inhibitor of metalloproteinase 2; letter c—spots 6203-1 and 6203-2 were analyzed separately by CapLC–MS/MS and matched to Pdc 109). Proteins were stained by Coomassie blue and standards spot numbers (SSP) were given to spots by PDQuest.

Table 1
Proteins in the accessory sex gland fluid of Holstein bulls identified by bidimensional SDS-PAGE and capillary liquid chromatography–nanoelectrospray ionization-tandem mass spectrometry (CapLC–MS/MS)

Protein identification[a]	GI accession number	Protein (kDa/p*I*)[b]		Mascot MS/MS score[c]	Seq. cov. (%)	Matched peptides and positions
		Theoretical	Experimental			
Osteopontin (spot 9802)	19774215	31; 4.5	55; 5.0	162	35	$^{(36)}$YPDAVATWLKPDPSQK$^{(51)}$; $^{(52)}$QTFLAPPQNSVSSEETDDNK$^{(70)}$; $^{(52)}$QTFLAPQNSVSSEETDDNKQNTLPSK$^{(77)}$; $^{(170)}$SNVQSPDATEEDFTSHIESEEMHDAPK$^{(196)}$; $^{(239)}$LSQEFHSLEDKLDLDHK$^{(255)}$; $^{(266)}$ISHELDASASEVN$^{(278)}$
BSP 30 kDa (Train 2)	28849953	22; 5.7				
Spot 7303			28; 5.1	17	3	$^{(159)}$VHSFFWR$^{(165)}$
Spot 7304			28; 5.0	56	9	$^{(159)}$VHSFFWR$^{(165)}$; $^{(167)}$WCSLTSNYDR$^{(176)}$
Spot 8302			28; 4.9	19	3	$^{(159)}$VHSFFWR$^{(159)}$
Spot 8303			28; 4.8	49	15	$^{(138)}$DEPECVPFIYR$^{(149)}$; $^{(159)}$VHSFFWR$^{(159)}$; $^{(167)}$WCSLTSNYDR$^{(176)}$;
Spot 8403			28; 4.7	36	12	$^{(138)}$DEPECVPFIYR$^{(149)}$; $^{(167)}$WCSLTSNYDR$^{(176)}$
Acidic seminal fluid protein (spot 9701)	3318757	13; 5.0	45; 4.3	48	31	$^{(57)}$ESLEIIDGLPGSPVLGK$^{(73)}$; $^{(96)}$EPEHPASFYEVLYFQDPQA$^{(114)}$
Acidic seminal fluid protein (spot 9101)	3318757	13; 5.0	13; 5.0	72	42	$^{(16)}$EESGVIATYYGPK$^{(28)}$; $^{(57)}$ESLEIIDGLPGSPVLGK$^{(73)}$; $^{(96)}$EPEHPASFYEVLYFQDPA$^{(114)}$
Acidic seminal fluid protein (spot 8101)	3318757	13; 5.0	13; 5.3	260	84	$^{(1)}$MDWLPR$^{(6)}$; $^{(16)}$EESGVIATYYGPK$^{(28)}$; $^{(29)}$TNCVMTIQMPPEYHVR$^{(44)}$; $^{(45)}$VSIQYLQLNCNK$^{(56)}$; $^{(57)}$ESLEIIDGLPGSPVLGK$^{(73)}$; $^{(74)}$ICEGSLMDYR$^{(83)}$; $^{(93)}$YIREPEHPASFYEVLYFQDPQA$^{(114)}$; $^{(96)}$EPEHPASFYEVLYFQDPA$^{(114)}$

Acidic seminal fluid protein (spot 6501)	3318757	13; 5.0	28; 5.8	124	61	(1)MDWLPR(6); (16)EESGVIATYYGPK(28); (45)VSIQYLQLNNK(56); (57)ESLEIIDGLPGSPVLGK(73); (93)YIREPEHPASFYEVLYFQDPQA(114); (96)EPEHPASFYEVLYFQDPQA(114)
Nucleobindin (spot 7801)	16758210	53.5; 5.0	58; 5.6	399	27	(53)YLQEVINVLETDGHFR(68); (136)QFEHLDPQNQHTFEAR(151); (152)DLELLIQTATR(162); (163)DLAQYDAAHHEEFKR(177); (189)YLESLGEEQR(198); (218)VNVPGSQAQLK(228); (229)EVWEELDGLDPNR(241); (310)LVTLEEEFLASTQR(322); (348)RFEEELAAR(356); (349)FEEEELAAR(356); (387)ELQQAVLQMEQR(398)
Clusterin precursor (spot 5901)	27806907	52; 5.7	70; 5.8	200	15	(52)TQIEQTNEER(61); (62)KLLLSSLEEAK(72); (63)LLLSSLEEAK(72); (153)IDSLMENDR(161); (177)ASSIMDELFQDR(188); (259)TPYHFPTMEFTENNDR(274); (332)LYDQLLQSYQQK(343)
Clusterin precursor (spot 6901)	116530	52; 5.7	70; 5.7	235	15	(52)TQIEQTNEER(61); (62)KLLLSSLEEAK(72); (63)LLLSSLEEAK(72); (153)IDSLMENDR(161); (177)ASSIMDELFQDR(188); (259)TPYHFPTMEFTENNDR(274); (332)LYDQLLQSYQQK(343)
Clusterin/Train 2	27806907	52; 5.7				
Spot 4701			40; 6.1	58	5	(177)ASSIMDELFQDR(188); (332)LYDQLLQSYQQK(343)
Spot 5603			40; 5.8	50	2	(177)ASSIMDELGQDR(188)
Spot 5604			40; 5.7	108	5	(177)ASSIMDELGQDR(188); (332)LYDQLLQSYQQR(343)

Table 1 (*Continued*)

Protein identification[a]	GI accession number	Protein (kDa/p*I*)[b]		Mascot MS/MS score[c]	Seq. cov. (%)	Matched peptides and positions
		Theoretical	Experimental			
Spot 6604			40; 5.6	328	20	$^{(63)}$LLLSSLEEAK$^{(72)}$; $^{(162)}$EQSHVMDVMEDSFTR$^{(176)}$; $^{(177)}$ASSIMDELFQDR$^{(188)}$; $^{(193)}$RPDQTQYYSPFSSFPR$^{(208)}$; $^{(209)}$GSLFFNPK$^{(216)}$; $^{(259)}$TPYHFPTMEFTENNDR$^{(274)}$; $^{(332)}$LYDQLLQSYQQK$^{(343)}$
Spot 6605			40; 5.5	273	22	$^{(63)}$LLLSSLEEAK$^{(72)}$; $^{(153)}$IDSLMENDREQSVMDVMEDSFTR$^{(176)}$; $^{(162)}$EQSHVMDVMEDSFTR$^{(176)}$; $^{(177)}$ASSIMDELFQDR$^{(188)}$; $^{(193)}$RPDQTQYYSPFSSFPR$^{(208)}$; $^{(209)}$GSLFFNPK$^{(216)}$; $^{(259)}$TPYHFPTMEFTENNDR$^{(274)}$; $^{(332)}$LYDQLLQSYQQR$^{(343)}$
Spot 6607			40; 5.4	187	16	$^{(63)}$LLLSSLEEAK$^{(72)}$; $^{(153)}$IDSLMENDREQSHVMDVMEDSFTR$^{(176)}$; $^{(177)}$ASSIMDELFQDR$^{(188)}$; $^{(259)}$TPYHFPTMEFTENNDR$^{(274)}$; $^{(332)}$LYDQLLQSYQQR$^{(343)}$
Bull seminal fluid protein Pdc 109 (spot 6203)	494430	5.6; 5.1	14; 5.7	107	64	$^{(5)}$CVFPFIYGGK$^{(14)}$; $^{(22)}$IGSMWMSWCSLSPNYDK$^{(38)}$; $^{(22)}$IGSMWMSWCSLSPNYDKDR$^{(40)}$
Bull seminal fluid Pdc 109 (spot 3301)	20663780	13; 5.1	14; 6.2	131	45	$^{(1)}$DQDEGVSTEPTQDGPAELPEDEECVFPFVYR$^{(31)}$; $^{(86)}$IGSMWMSWCSLSPNYDK$^{(102)}$; $^{(86)}$IGSMWMSWCSLSPNYDKDR$^{(104)}$
Spermadhesin Z13 (Train 5)	12585540	14; 5.6				
Spot 1201			14; 6.8	100	25	$^{(61)}$ESLEIIEGPPESSNSR$^{(76)}$; $^{(100)}$KPNHPAPDFFLIFR$^{(113)}$
Spot 2201			14; 6.5	111	25	$^{(61)}$KPNHPAPDFPLIFR$^{(76)}$; $^{(100)}$ESLEIIEGPPESSSNSR$^{(113)}$

Spot 2202			14; 6.3	154	33	$^{(1)}$DSTDGLLVK$^{(1)}$; $^{(61)}$KPNHPAPDFFLIFR$^{(76)}$; $^{(100)}$ESLEIIEGPPESSNSR$^{(113)}$
Spermadhesin Z13 (spot 3601)	12585540	14; 5.6	29; 6.3	60	25	$^{(61)}$KPNHPAPDFFLIFR$^{76)}$; $^{(100)}$ESLEIIEGPPESSNSR$^{(113)}$
TIMP-2/Train 4	27806163	25; 7.5				
Spot 502			26; 7.2	53	13	$^{(54)}$EVDSGNDIYGNPIK$^{(67)}$; $^{(206)}$GAAPPKQEFLDIEDP$^{(220)}$
Spot 1501			25; 7.1	246	28	$^{(28)}$EVDSGNDIYGNPIK$^{(41)}$; $^{(28)}$EVDSGNDIYGNPIKR$^{(42)}$; $^{(90)}$AEGNMHITLCDFIVPWDTLSATQK$^{(115)}$; $^{(180)}$GAAPPKQEFLDIEDP$^{(194)}$; $^{(186)}$QEFLDIEDP$^{(194)}$
Spot 1502			25; 6.8	150	8	$^{(54)}$EVDSGNDIYGNPIK$^{(67)}$; $^{(54)}$EVDSGNDIYGNPIKR$^{(68)}$
Spot 2502			25; 6.7	155	13	$^{(54)}$EVDSGNDIYGNPIK$^{(67)}$; $^{(54)}$EVDSGNDIYGNPIKR$^{(68)}$; $^{(206)}$GAAPPKQEFLDIEDP$^{(220)}$
Cathepsin L (spot 1601)	27806673	38; 6.5	38; 6.9	68	12	$^{(39)}$LYMNEEEWR$^{(48)}$; $^{(58)}$IIDLHNQEYSEGK$^{(70)}$; $^{(75)}$MAMNAFGDMTNEEFR$^{(89)}$
Ecto-ADP-ribosyltransferase 5 (spot 2702)	9998751	27; 7.6	43; 6.3	199	27	$^{(64)}$EMADHALLR$^{(72)}$; $^{(86)}$RPGLTLPPGFR$^{(96)}$; $^{(123)}$TGGGSWESYMNHFPFK$^{(138)}$; $^{(139)}$ALHFYLTR$^{(146)}$; $^{(160)}$EPGQVVFR$^{(167)}$; $^{(185)}$LGQFTSSSLDETVAR$^{(199)}$
Abumin (spot 2901)	162648	71; 5.8	63; 6.4	178	7	$^{(402)}$HLVDEPQNLIK$^{(412)}$; $^{(421)}$LGEYGEQNALIVR$^{(433)}$; $^{(548)}$KQTALVELLK$^{(557)}$; $^{(569)}$TVMENFVAFVDK$^{(580)}$
PLA_2 (Train 1)	27807045	51; 6.1				
Spot 3801			58; 6.2	192	13	$^{(42)}$IQALMAAANIGQSK$^{(55)}$; $^{(110)}$FLGTHWLVGK$^{(119)}$; $^{(144)}$YPLIIFSHGLGAFR$^{(157)}$; $^{(311)}$IPQPLFFINSER$^{(322)}$; $^{(365)}$IIGYLFTLK$^{(373)}$

Table 1 (*Continued*)

Protein identification[a]	GI accession number	Protein (kDa/p*I*)[b]		Mascot MS/MS score[c]	Seq. cov. (%)	Matched peptides and positions
		Theoretical	Experimental			
Spot 4801			58; 6.1	106	9	$^{(42)}$IQALMAAANIGQSK$^{(55)}$; $^{(110)}$FLGTHWLVGK$^{(119)}$; $^{(211)}$RGEEEFPLR$^{(219)}$; $^{(323)}$FQYPSNIIR$^{(331)}$
Spot 4802			58; 6.0	172	20	$^{(42)}$IQALMAAANIGQSK$^{(55)}$; $^{(102)}$EYFLGLSK$^{(109)}$; $^{(110)}$FLGTHWLVGK$^{(119)}$; $^{(144)}$YPLIIFSHGLGAFR$^{(157)}$; $^{(268)}$IAIIGHSFGGATVIQTLSEDQR$^{(289)}$; $^{(311)}$IPQPLFFINSER$^{(322)}$; $^{(323)}$FQYPSNIIR$^{(331)}$
Ecto 5′-nucleotidase (spot 1703)	27806507	64; 6.4	63; 6.8	124	11	$^{(134)}$EVNFPILSANIK$^{(145)}$; $^{(163)}$ILTVGDEVVGIVGYTSK$^{(179)}$; $^{(257)}$EVPAGQYPFIVTSDDGR$^{(273)}$; $^{(274)}$KVPVVQAYAFGK$^{(285)}$; $^{(537)}$VLYPAVEGR$^{(545)}$

TIMP-2: tissue inhibitor of metalloproteinase 2; PLA_2: phopholipase A_2.

[a] Spot and train # as shown in 2D maps of Fig. 1A and B.

[b] Theoretical molecular weight and p*I* values represent the values given for the protein identified at NCBI NR database. Experimental values were deduced from the 2D master gel generated by PDQuest.

[c] Individual ion scores ≥48 indicate identity or extensive homology ($p < 0.05$). The protein score represents the sum of the unique ion scores when more than one precursor ion was used for MS/MS analysis.

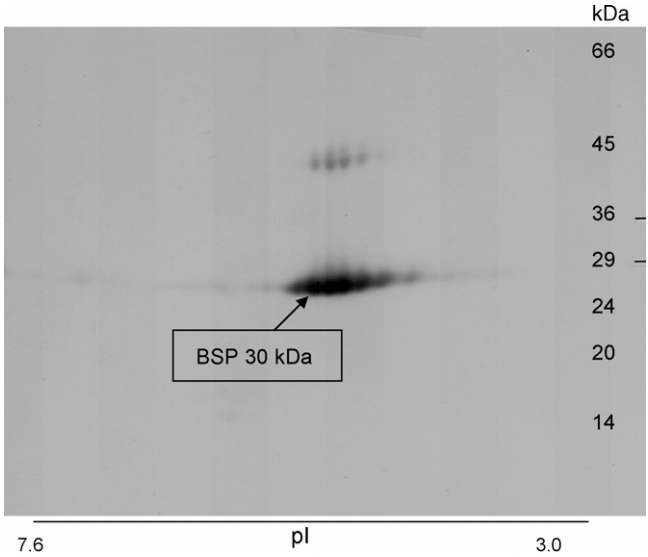


Fig. 3. Western blot of accessory sex gland fluid proteins incubated with anti-BSP 30 kDa. Proteins were separated by 2D SDS-PAGE and transferred to nitrocellulose membranes.

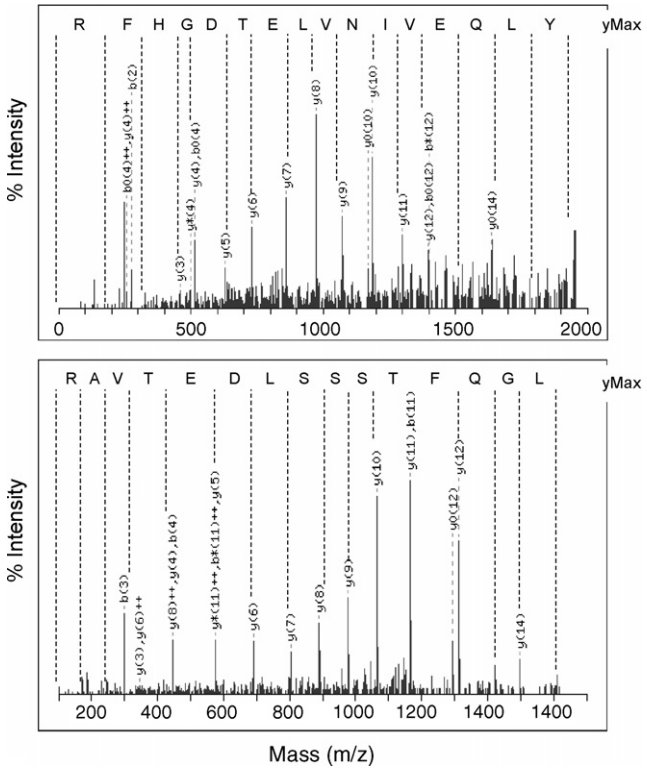


Fig. 4. Product ion spectrum of trypic peptides YLQEVINVLETDGHFR and LGQFTSSSLDETVAR matched by MASCOT to nucleobindin (panel A) and ecto-ADP-ribosyltransferase 5 (panel B), respectively.

We were able to identify the majority of the proteins visualized in the AGF 2D maps. A core group of 20 spots representing clusterin, albumin, nucleobindin, osteopontin, acidic seminal fluid protein, TIMP-2, spermadhesin Z13 and BSP proteins (BSP 30 kDa and BSP A1/A2 and A3) was expressed in all 37 bulls, but with distinct degrees of variability. The amount of BSP A1/A2 and A3, TIMP-2, spermadhesin and nucleobindin had less variation across bulls (CV $\leq$ 52.1%) while osteopontin, clusterin and aSFP showed the highest variability. Moreover, proteins such as nucleotidase and ART-5 were detected only in a small number of animals (9 and 7 bulls, respectively). This degree of variation probably suggests the existence of complex mechanisms by which the synthesis of AGF proteins is controlled. It should also be considered that AGF proteins could be related to other endpoints besides field fertility scores.

Several proteins appeared as multiple spots, which may have been generated by a diverse range of post-translational modifications. Although it was not our intention to determine post-translational modifications responsible for each series of spots in the gels, the literature suggests that glycosylation is the primary reason for appearance of 40 kDa clusterin (Sensibar et al., 1993) and BSP 30 kDa (Manjunath and Thérien, 2002) isoforms. In general it is believed that phospholipase A_2 isoforms are the result of phosphorylation, farnesylation and carboxymethylation (Jenkins et al., 2003). The multiple spots associated with spermadhesin Z13 and aSFP are believed to be subunits of the respective proteins detected in gels with β-mercaptoethanol (Einspanier et al., 1993; Tedeschi et al., 2000).

A few spots in the AGF 2D maps could not be identified possibly because of the low amount of protein recovered from the spots. We are also aware that the combination of ampholytes for electrofocusing that we used along with the Coomassie stain unquestionably favored the detection of the most abundant proteins in the samples. Therefore, one cannot exclude the possibility that detection of some less abundant proteins that co-migrated with the major proteins may have been missed. Despite these facts, we believe that the use of a broad pH range in the gels was appropriate given that this is the first proteomic analysis of a composite fluid obtained from the bull accessory sex glands.

Most proteins we identified in the AGF have the capacity to interact to cauda epididymal sperm when ejaculation occurs, given their known attributes. This raises the possibility that some AGF proteins remain associated with sperm and influence events occurring in the female reproductive tract associated with sperm transport, capacitation and fertilization. A discussion of how known functions of proteins detected in the AGF may potentially impact sperm physiology is presented in the following sections and summarized in Fig. 5.

4.1. Accessory sex gland fluid proteins that participate in sperm capacitation

Capacitation is one of the early events occurring after the sperm is mixed with AG secretions. The complex of BSP proteins (A1/A2, A3 and BSP 30 kDa) present in the AGF are known to induce cholesterol efflux from the sperm membrane (Manjunath and Thérien, 2002) and, along with its ability to mediate sperm capacitation, BSP A1/A2 binds to epithelial cells of the oviduct and appears to contribute to the formation of a sperm reservoir (Gwathmey et al., 2003). Our earlier studies have shown that BSP 30 kDa expressed in the AGF had a quadratic association with bull fertility indexes (Moura et al., 2006). We suggested that this type of association may be linked to BSP's ability to modulate cholesterol efflux from sperm membrane and potential interaction with phospholipase A_2. Albumin, another AGF protein that binds to sperm, may also influence capacitation through its ability to modulate membrane cholesterol (Go and Wolf, 1985; Visconti and Kopf, 1998).

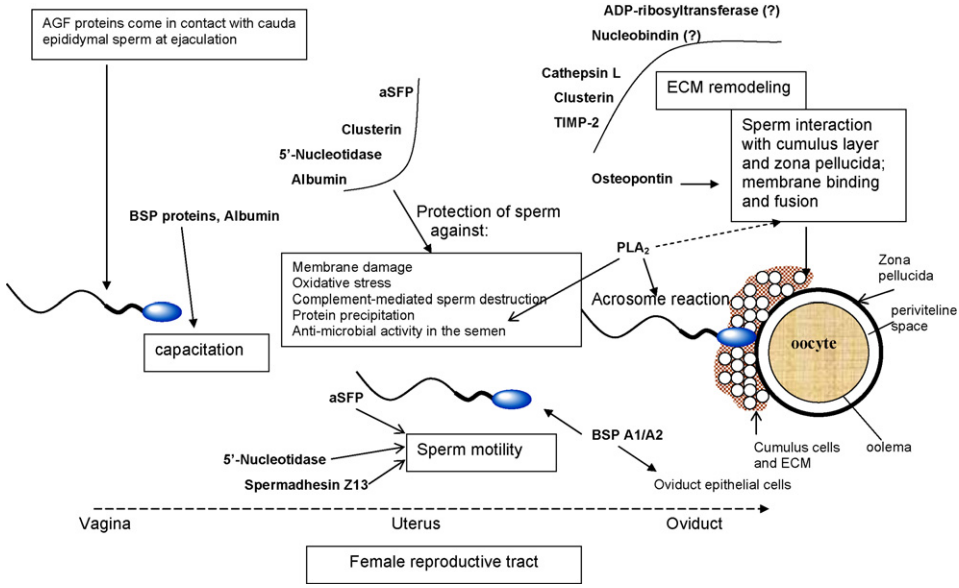


Fig. 5. Schematic diagram of aspects of sperm physiology and fertilization that are potentially influenced by the proteins identified in the accessory sex gland fluid of mature Holstein bulls. After ejaculation, binding of BSP proteins and albumin contribute to capacitation. In the female reproductive tract, protection of sperm against oxidative stress, damage caused by hydrophobic molecules, protein precipitation and complement-induced attack could be mediated by AGF proteins such as aSFP, clusterin, 5′-nucleotidase and albumin. When the sperm comes in contact with the cumulus layer, interaction with and remodeling of the extracellular matrix (ECM) must be of importance. It is feasible thus that proteins such as TMIP-2, clusterin and possibly cathepsin L participate in these events. PLA_2 secreted into the AGF acts during acrosome reaction, membrane fusion and has reported antimicrobial activity. Sperm-oocyte interaction also involves osteopontin. Sperm motility, a "sine qua non" for fertilization to occur in vivo, can be affected by aSFP, spermadhesin Z13 and 5′-nucleotidase. The roles of nucleobindin and ADP-ribosyltransferase, two new proteins detected in the AGF, are still unknown.

4.2. *Proteins involved in protection of sperm*

The accessory sex gland fluid contains proteins that intervene, either directly or indirectly, in mechanisms aimed to prevent damage to the sperm membrane, oxidative stress and immune attack. Albumin absorbs lipid peroxides, which contributes to its protective effect on both sperm membrane and motility (Alvarez and Storey, 1995) and aSFP is known to inhibit oxidative stress (Einspanier et al., 1993; Schoneck et al., 1996). Bovine aSFP shares identity with proteins of the spermadhesin family (Romão et al., 1997). Binding of aSFP to ejaculated sperm occurs but it is lost after capacitation (Dòstolovà et al., 1994), suggesting that, unlike porcine spermadhesins (Caballero et al., 2004, 2005), bovine aSFP does not participate in sperm-oocyte interaction. The aSFP isoforms we found in the AGF (p*I*s at 5 and 5.3), including the spot at 28 kDa, are similar to the aSFPs that appear in the bovine seminal plasma at 13 kDa and a dimer at 26 kDa (Einspanier et al., 1994; Romão et al., 1997). However, the 45 kDa aSFP identified in the AGF has not been previously described in the seminal plasma. It is possible that aSFP isoforms in AGF have different characteristics from those observed in the seminal milieu.

The 70 and 40 kDa isoforms of clusterin in the AGF maps are similar to those reported in the cauda epididymis fluid of bulls (Ibrahim et al., 1999). Clusterin is a multifunctional constituent

of the accessory sex gland secretions and it can prevent oxidative damage to the sperm (Reyes-Moreno et al., 2002), bind and agglutinate abnormal spermatozoa in bulls (Ibrahim et al., 1999) and humans (O'Bryan et al., 1990, 1994) and act like a chaperone, protecting sperm from the toxic effects of protein precipitation (Humphreys et al., 1999; Wilson and Easterbrook-Smith, 2000). Clusterin has the ability to inhibit complement-induced sperm lysis (Jenne and Tchopp, 1989; O'Bryan et al., 1990; Ibrahim et al., 1999) and this is also one of the features of the 5′-nucleotidase enzyme (Takayama et al., 2000) found in the AGF. Protection of sperm from oxidative damage, agglutination or lysis could be important for the spermatozoa once in the female reproductive tract.

4.3. Proteins involved in the acrosome reaction and sperm-oocyte interaction

Phospholipase A_2 (PLA_2) has a diverse range of functional features some of which relate to the acrosome reaction and sperm-oocyte membrane fusion (Riffo and Párraga, 1997; Yuan et al., 2003). However, there is also evidence that PLA_2 stimulates immune cells (Granata et al., 2005) and has antimicrobial activity in the seminal plasma (Weinrauch et al., 1996; Bourgeon et al., 2004). Osteopontin is another multifunctional protein identified in the AGF of bulls and it was originally described in the extracellular matrix of bone tissues (Senger et al., 1979). Osteopontin interacts with the membrane of ejaculated bull sperm and also affects sperm-oocyte binding and early embryonic development (our unpublished results). We previously reported that the expression of both PLA_2 and osteopontin was more pronounced in the accessory sex gland fluid of high fertility bulls than in low fertility bulls (Moura et al., 2006). A more in depth discussion of their attributes as related to male fertility was presented therein.

4.4. Proteins potentially associated with interaction and modulation of extracellular matrix (ECM) components

Interaction and remodeling of the ECM are some of the attributes of proteins such as TIMP-2, clusterin and probably cathepsin L, found in the accessory sex gland fluid. This group of AGF proteins may be important during mammalian fertilization when the sperm interact with and cross barriers established by the cumulus cells, zona pellucida and oocyte membrane. Metalloproteinases (MMPs) released from the sperm during acrosome reaction facilitate sperm-egg fusion in hamsters (Díaz-Perez and Meizel, 1992). TIMP-2 associates with bovine and human sperm membranes (McCauley et al., 2001; Bechman-Shaked et al., 2002) and inhibitors of MMPs and TIMPs interfere with gamete fusion in mice (Correa et al., 2000). Clusterin interacts with ECM complexes in the testis (Sylvester et al., 1991; Bailey and Griswold, 1999) and a more recent study found that high molecular weight clusterin (70–73 kDa) inhibits certain types of metalloproteinases (MT6-MMP/MMP-25) produced by neutrophils (Matsuda et al., 2003), a role shared by other controllers of MMPs. The cathepsin L detected in AGF has the same molecular weight as a procathepsin L molecule found in the acrosome of epididymal spermatozoa (McDonald and Emerick, 1995). Cathepsins are cysteine peptidases involved in proteolysis of components usually found as part of the ECM during growth, tissue invasion and remodeling (Dickinson, 2002).

4.5. Proteins associated with sperm motility

While several biochemical changes occur in the male gametes from emission until fertilization, sperm must also traverse the female reproductive tract to reach the oocyte. This is accomplished

by a combination of contractions of the female reproductive tract and sperm motility. Some accessory sex gland proteins have the potential to influence sperm motility, including BSP A1/A2 (Sanchez-Luengo et al., 2004), aSFP (Schoneck et al., 1996) and phospholipase A_2 (Bao et al., 2004). Interestingly, bovine seminal plasma aSFP shares a 50% homology with spermadhesin Z13 (Tedeschi et al., 2000), the second most abundant group of proteins that we were able to detect in the AGF 2D maps. We showed that this AGF spermadhesin had an inverse relationship with fertility (Moura et al., 2006) but the mechanism for its affect on sperm in the bull is unclear. The ecto 5′-nucleotidase (5′-NT) is another AGF component that can be grouped in this category because antibodies against and inhibitors of this enzyme are associated with reductions in human spermatozoa motility in vitro (Aumuller et al., 1997). This enzyme causes hydrolysis of nucleoside 5-monophosphates (Thompson, 1985) and it has been shown as part of the bovine (Schiemann et al., 1994) and human spermatozoa (Takayama et al., 2000).

4.6. New proteins expressed in the accessory sex gland fluid

The ecto-ADP-ribosyltransferase 5 (ART 5) identified in the AGF belongs to a class of enzymes that catalyze the transfer of an ADP-ribose group from NAD to arginine residues on target proteins, such as G proteins, rho, actin, CD44 and α integrins (Okazaki and Moss, 1999; Mueller-Dieckmann et al., 2002; Seman et al., 2004). ARTs (ADP-ribosyltransferases) are normally expressed in immune cells and ART5, in the testis (Mueller-Dieckmann et al., 2002; Corda and Di Girolamo, 2003), but none of these forms had been reported before in secretions of accessory sex glands. Another unique spot in the AGF two-dimensional gels was identified as nucleobindin, a protein with Ca^{2+} and DNA-binding motifs and conserved among species (Wendel et al., 1995). To our knowledge, this is the first identification of nucleobindin as a secreted component of the accessory sex gland fluid or seminal plasma. Interestingly, nucleobindin has been found secreted by bone cells and odontoblasts and as a structural element of their respective extracellular matrices (Petersson et al., 2004; Somogyi et al., 2004). These features resemble those of osteopontin, another bone protein that was identified in the bull seminal plasma and accessory sex gland fluid by our laboratory (Killian et al., 1993; Cancel et al., 1997; Moura et al., 2006).

5. Conclusion

Using a systematic proteomic analysis of 37 different 2D maps, we obtained a global view of different classes of proteins expressed in the accessory sex gland fluid of proven, high use Holstein bulls with documented fertility history. The recognized characteristics of the AG proteins suggest they modulate important sperm functions after ejaculation and in the female reproductive tract. Some of these proteins are described for the first time as part of the accessory sex gland fluid. These analyses and observations provide justification for studies to explore and better understand the mechanisms by which accessory sex gland proteins influence male fertility.

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