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Purification and Characterization of Fertility-Associated Antigen (FAA) in Bovine Seminal Fluid

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ABSTRACT Heparin-binding proteins (HBP) recognized by a monoclonal antibody (M1) are produced by male accessory sex glands and bind to distinct regions of ejaculated bull sperm. Immunoblots of sperm proteins probed with M1 identified HBP variants of approximately 31-, 24-, and 21.5-kDa that were associated with increased fertility of bulls. The purpose of this study was to identify the 31-kDa HBP known as fertility-associated antigen (FAA). FAA was isolated by heparin-affinity chromatography and reversed-phase high performance liquid chromatography near homogeneity. Biochemical characterization indicated that FAA was an unglycosylated, basic protein. FAA protein was detected in seminal vesicle and prostate gland homogenates, and FAA extracted from sperm membranes by treatment with hypertonic media was identical biochemically to seminal fluid-derived FAA. N-terminal sequence analysis of purified FAA yielded a 26 amino acid sequence (L K I X S F N V R S F G E S K K A G F N A M R V I V) with 73% identity to a recently identified human deoxyribonuclease (DNase) I-like protein. Two internal amino acid sequences generated from lys-C digested FAA were 85% and 92% identical to the same DNase I-like protein. In conclusion, we have identified a bovine seminal heparin-binding protein that binds to sperm and is indicative of bull fertility as being similar to the family of DNase I-like proteins. These data demonstrate the presence of a novel DNase I-like protein in bull accessory sex glands and form the groundwork for the identification of a candidate genetic marker for fertility of bulls. *Mol. Reprod. Dev. 54:145–153, 1999.*

Key Words: accessory sex glands; bull; deoxyribonuclease; fertility; sperm

INTRODUCTION

The addition and removal of a variety of proteins and lipid constituents during epididymal maturation and at ejaculation play an important role in capacitation of sperm (Saling, 1989; Yanagimachi, 1994). Bull accessory sex glands secrete heparin-binding proteins (HBP) as a prominent component of seminal fluid. Binding of HBP and heparin to cauda epididymal sperm in vitro stimulated increased frequencies of zonae pellucidae-induced acrosome reactions (Miller et al., 1990). Five classes of HBP with progressively higher affinity for heparin were identified. Those classes consisted of HBP complexes ranging in molecular weight from 14 to 31 kDa, with the 31-kDa protein predominant in complexes with the greatest affinity for heparin (Miller et al., 1990).

Heparin binding to sperm has been well documented in a variety of species. Heparin binds to bull, rabbit, monkey (Handrow et al., 1984), boar (Sanchez-Prieto et al., 1996), and human sperm (Delgado et al., 1982; Miller et al., 1988). The interaction between heparin and sperm is an important indicator of fertility potential of bulls. Sperm from higher fertility bulls bound heparin with higher affinity (Marks and Ax, 1985) and underwent increased rates of acrosome reaction in response to treatment with heparin-like glycosaminoglycans compared to lower fertility bulls (Ax et al., 1985; Lenz et al., 1988).

Heparin-binding proteins originating from the accessory sex glands modulate heparin-sperm interactions that are indicative of fertility. Presence of three molecular weight HBP variants on sperm was associated with increased fertility potential of bulls (Bellin et al., 1996, 1998). One of those variants, an HBP of ~31 kDa was recently described as fertility-associated antigen (FAA; Bellin et al., 1998). FAA was present on sperm from bulls which consistently impregnated a greater number (9 percentage points) of cows compared to bulls without the protein on sperm, regardless of presence or absence of the other two HBP variants. Characterizing functionally important HBP such as FAA is a first step toward better understanding the modulating effects of seminal fluid on fertility of bulls. In the present study we describe purification of FAA, characterize its interaction with sperm membranes, present partial amino acid sequence of purified FAA, and examine FAA production in bovine accessory sex glands.

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MATERIALS AND METHODS

Isolation of Fertility-Associated Antigen (FAA)

Fertility-associated antigen (FAA) was isolated from bovine seminal fluid, sperm membranes, and accessory sex glands. Seminal fluid was collected by artificial vagina from a vasectomized Holstein bull housed at Sire Power, Inc. (Tunkhannock, PA). That bull was shown prior to vasectomy to produce the 31-kDa protein of interest. Seminal fluid was frozen in liquid nitrogen and shipped to the University of Arizona. After thawing, seminal fluid was centrifuged at 12,000*g* for 5 min at 4°C. Five-hundred microliters to 1 ml of seminal fluid supernatant (sperm KCl extracts or accessory sex gland proteins; described below) was applied to a heparin-affinity column (heparin econo-pac, Bio-Rad, Hercules, CA; or heparin-sepharose CL-6B, Pharmacia [5 × 250 mm]) connected in-line to a peristaltic pump (ISCO, Inc., Lincoln, NE) at a flow rate of approximately 1 ml/min. The column was equilibrated with 40 mM Tris (pH 7.4), 2 mM $CaCl_2$, 200 µM PMSF, 0.01% NaN_3 (TC-A). Peak detection was achieved by monitoring absorbance at 280 nm with an UA-5 absorbance detector (ISCO, Inc.). After non-binding proteins had cleared the column and baseline conditions were achieved, HBP were eluted with 2 M NaCl in TC-A. The fraction containing FAA was diluted in TC-A to 15 ml and desalted/concentrated by centrifugation (Beckman J-6M, 2,000*g*) in centriprep tubes (Ultrafree-15, 12,000 MWCO, Millipore, Bedford, MA). All separations were performed at 4°C. Samples were assayed to determine protein concentration (Bio-Rad Dc protein assay, Bio-Rad) using bovine serum albumin (bSA) as standard. Desalted fractions were immediately frozen and stored lyophilized until further analysis.

Extraction of FAA From Sperm

Cryopreserved semen from bulls of proven fertility (generously provided by Select Sires, Plain City, OH) was thawed at 37°C and centrifuged at 300*g* for 10 min. The sperm pellet was washed 3× in phosphate-buffered saline (PBS) with protease inhibitors (10 µM leupeptin, 1 µM pepstatin A, 200 µM PMSF, pH 7.4). An aliquot of non-extracted, washed ejaculated sperm was solubilized in electrophoresis sample buffer. The remainder of the sample was resuspended in PBS, with or without (control) 0.6 N KCl, and gently agitated for 60 min at 4°C. The sperm suspension was centrifuged (600*g*, 10 min), and the supernatant containing the KCl extract (or control proteins) was centrifuged at 14,000*g* for 20 min. The clarified supernatant was concentrated and desalted by centrifugation as described above or by dialysis (10,000 MWCO), assayed for protein concentration, and solubilized in electrophoresis sample buffer. Extracted sperm were washed 3× in PBS to remove excess salt and solubilized in electrophoresis sample buffer. In addition, the same extraction procedure was repeated on a larger scale and sperm extracts were fractionated by heparin-affinity chromatography and RP-HPLC as described below.

Accessory Sex Gland Preparation

To determine the origin of FAA synthesis, bovine accessory sex glands (seminal vesicles, prostate, and bulbourethral gland) were obtained immediately after slaughter and snap frozen in liquid nitrogen. Tissues were dissected and rinsed in cold buffer (50 mM Tris-Cl (pH 7.5) 150 mM NaCl, 2 mM EDTA, 0.5 mM DTT) and mechanically homogenized on ice in 50 mM Tris-Cl (pH 7.5), 1% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF. Total homogenates from each gland were centrifuged at 14,000*g* for 60 min and supernatants were extracted by addition of saturated $(NH_4)_2SO_4$. Following centrifugation, protein pellets were resuspended in PBS with protease inhibitors, dialyzed, lyophilized and subjected to heparin-affinity chromatography and RP-HPLC as described below.

Reversed-Phase HPLC

Lyophilized powder was resuspended in buffer A (95% H_2O/5% acetonitrile in 0.1% (wt:vol) trifluoroacetic acid [TFA]). Preliminary RP-HPLC profiles were used to calculate the percentage of acetonitrile required to elute FAA from a C4 matrix. Buffers containing percentages of acetonitrile below and above the critical elution concentration (44% acetonitrile) for FAA were used in solid-phase extraction (SPE) of HBP with 2-ml C4 disposable columns (Vydac, Hesperia, CA). After SPE, HBP (100 µl to 1 ml) were injected by a Hitachi HPLC autosampler onto a C4 reversed-phase HPLC column (Vydac). Proteins were fractionated with a multi-step linear gradient from 25% buffer A to 100% buffer B (70% acetonitrile in 0.085% (wt:vol) TFA) over 55 min, with a total run time of 60 min. Thirty-second fractions (1.5 ml/fraction) were collected using a Foxy fraction collector (ISCO, Inc.) linked to a diode array detector, and dried by rotary evaporation (Speedvac, Savant Instruments, Farmingdale, NY).

SDS-PAGE and Immunoblotting

A portion of each reversed-phase HPLC fraction was analyzed by discontinuous SDS-PAGE and Western blotting using a monoclonal antibody, M1, raised in mice immunized with purified HBP (Bellin et al., 1996). Dried fractions were resuspended in sample buffer and solubilized by boiling for 5 minutes. One-dimensional SDS-PAGE was performed according to the method of Laemmli (1970). Ten microliters of prestained molecular weight markers phosphorylase B (101 kDa), BSA (83 kDa), ovalbumin (50.6 kDa), carbonic anhydrase (35.5 kDa), soybean trypsin inhibitor (29.1 kDa), and lysozyme (20.9 kDa; Bio-Rad Laboratories) were applied to one lane. Samples were electrophoresed through an 8 × 10 cm 13.5% polyacrylamide gel (Mighty Small II, Hoefer Scientific Instruments, San Francisco, CA) for approximately 90 min at 20 mA constant current per gel. Proteins were then transferred (1 hr at 150 mA constant current) using a semi-dry electroblotter (Millipore Milliblot Graphite Electroblotter I) to a 0.2 µm polyvinylidene difluoride (PVDF) membrane (Trans-

blot, Bio-Rad; or Immobilon P^{sq}, Millipore) using 10 mM 3-cyclohexylamino-1-propane-sulfonic acid (CAPS) in 10% MeOH as electroblotting buffer. Two identical gels were run in parallel; one was stained with coomassie blue and the other was used for immunoblotting or for microsequencing. For microsequencing, PVDF was stained with Ponceau S (0.2% Ponceau S in 1% acetic acid) and FAA was excised. Immunostaining with M1 was performed as previously described (McCauley et al., 1996). Blotted membranes were blocked with 5% BSA in PBS-T (PBS with 3% Tween) prior to incubation with M1 hybridoma culture supernatant (1:10 in PBS-T). Membranes were rinsed 3× with PBS-T, and goat-anti mouse IgG horseradish peroxidase conjugate (1:4000; BioSource International; Camarillo, CA) was added for 60 min. Experiments were performed in the absence of M1 (secondary alone) as a negative control. Western blots were developed by enhanced-chemiluminescence (ECL; Amersham, Northbrook, IL) and exposed on x-ray film (Eastman Kodak, Rochester, NY). Images of stained acrylamide gels and Western blots were captured and analyzed with AlphaImager Digital Analysis software (Alpha Innotech Corp., San Leandro, CA).

Two-Dimensional Electrophoresis

The RP-HPLC fraction containing FAA was subjected to 2-D electrophoresis essentially as described by O'Farrell, (1975). FAA was solubilized in lysis buffer (9 M urea, 2% NP-40, 5% 2-mercaptoethanol, and ampholines). Ampholytes (pH 5–8 and pH 3–10) were mixed to establish a final pH gradient ranging from 4.5 to 8.5, as determined by measuring pH of 5 mm segments of tube gel. Samples were loaded into IEF tube gels (SE 220 Tube Gel Adapter Kit, Hoefer Scientific Instruments) and electrofocused at 500 V for 2.5 hr using 20 mM NaOH and 10 mM H_3PO_4 as electrode solutions. Focused gels were placed on a 5% stacking gel, overlayered with tracking dye, and electrophoresed through a 13.5% separating gel. One lane included molecular weight markers as used in one-dimensional SDS-PAGE. The gel was stained with coomassie blue following second dimension separation.

Glycoprotein Analysis

Protein glycosylation was detected using an immunoblot-based glycoprotein detection kit (Immun-Blot, Bio-Rad) according to the procedure recommended by the manufacturer to label carbohydrates on PVDF membranes. Briefly, HPLC fractions were separated by SDS-PAGE and transferred to a PVDF membrane as described above. The membrane was washed 3× for 10 min with 50 ml of PBS (pH 7.2) and immersed in 50 ml of 10 mM sodium periodate, 5 mM EDTA, 100 mM sodium acetate solution (pH 5.5) in the dark for 20 min. After washing in PBS, the membrane was placed in 50 ml of 100 mM sodium acetate/5 mM EDTA containing 2 µl of biotinylated hydrazide solution and incubated for 60 min. After washing in Tris-buffered saline (TBS, pH 7.2), the membrane was incubated for 1 hr at room temperature or overnight at 4°C with 50 ml of blocking reagent supplied by the vendor. After washing in TBS, the membrane was immersed in streptavidin-alkaline phosphatase conjugate, diluted in TBS, for 1 hr. Finally, after washing, the membrane was incubated with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) substrate to visualize carbohydrate containing samples.

N-Terminal Amino Acid Sequencing

Amino acid analysis and N-terminal protein sequencing were performed at the University of Arizona Macromolecular Structure Facility. Sequence analysis was performed using an Applied Biosystems 477A Protein/Peptide Sequencer interfaced with a 120A HPLC Analyzer (C-18 PTH column). Total amino acid analysis was determined with a dedicated Applied Biosystems Model 420A Amino Acid Analyzer with automatic hydrolysis (vapor phase at 160°C for 100 min using 6N HCl) and pre-column with phenylthiocarbamyl-amino acid. N-terminal amino acid sequencing of internal peptides generated by lys-C digestion of purified FAA was performed by the Protein Chemistry Core at Baylor College of Medicine. Polyacrylamide gel-purified FAA was briefly stained with coomassie blue (0.05% coomassie in 10% methanol/5% acetic acid) and cored from the gel. In-gel lys-C enzymatic digestion was performed essentially as described by Rosenfeld et al. (1992). Briefly, 1–2 mm gel pieces were rinsed 30–60 min in 0.1 M Tris (pH 8.0), 50% acetonitrile, dried, and rehydrated in 100 µl digestion buffer including 1 µg lys-C. Samples were digested for 24 hr and peptides were extracted by sonication in 60% acetonitrile, 0.1% TFA. Extracted peptides were analyzed with a Pharmacia SMART HPLC System equipped with a microbore column. Sequence analysis was performed on selected peptide fractions using PE Applied Biosystems sequencing instrumentation. Similarity searches were performed using the Blast programs (Altschul et al., 1997) of the National Center for Biotechnology Information at the National Library of Medicine.

RESULTS

Isolation of Fertility-Associated Antigen (FAA)

Purification of seminal FAA to near homogeneity was accomplished with a combination of heparin-affinity, solid-phase extraction, and reversed-phase high performance liquid chromatography (RP-HPLC). Densitometric analysis indicated a 175-fold purification of FAA was achieved. Approximately 20 mg of HBP was isolated per ml of seminal fluid collected from a vasectomized bull. Approximately 0.4% of the total protein was identified as FAA (Table 1). HBP were fractionated with C4 SPE columns and subsequently injected onto an RP-HPLC C4 column. A typical HPLC profile of seminal HBP fractionation is shown in Figure 1A. The protein elution profile indicated FAA was hydrophobic, requiring 44% acetonitrile (26.8 min peak) to displace it from the column.

TABLE 1. Purification of Fertility-Associated Antigen (FAA) From Bull Seminal Fluid

Step[a]	Sample[b]	Total protein (mg/ml)	Protein recovered (%)	FAA recovered (mg/ml)	% FAA	-Fold purification of FAA
—	VSF	46.7 ± 2.0[c]	100	—	0.36	—
HA	HBP	21 ± 1.5[c]	45	—	0.81	2.3
RP-HPLC	PEAK	0.27[d]	0.57	0.17[e]	63	175

[a]HA, heparin affinity chromatography; RP-HPLC, reversed-phase high performance liquid chromatography.
[b]VSF, seminal fluid from a vasectomized bull; HBP, bound fraction from heparin affinity chromatography; PEAK, fraction eluting from HPLC at 26.8 min.
[c]Quantified by Bio-Rad Dc microtiter protein assay.
[d]Determined from integrated area under peak quantified by HPLC.
[e]Calculated by densitometric analysis of FAA following SDS-PAGE.

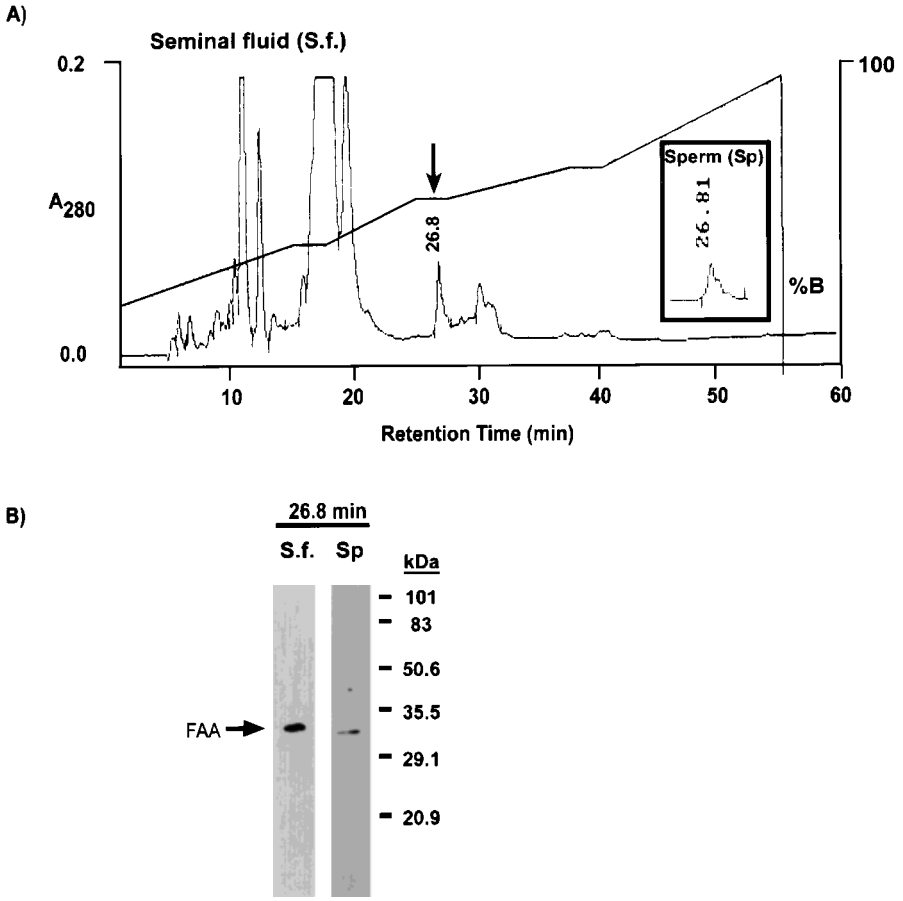

Fig. 1. Reversed-phase high performance liquid chromatography (RP-HPLC) of bovine heparin-binding proteins from seminal fluid and sperm membranes and identification of fertility-associated antigen (FAA). (**A**) RP-HPLC separation of heparin-binding proteins isolated from bovine seminal fluid (S.f.) or from sperm extracts (insert; Sp). Proteins were isolated by heparin-affinity chromatography and fractionated by RP-HPLC using a gradient from 5.5% acetonitrile/0.1% TFA to 70% acetonitrile/TFA in 55 min. A representative chromatogram is shown. (**B**) Western blot of RP-HPLC peak eluting at 26.8 min. HPLC fractions were analyzed by SDS-PAGE and transferred to PVDF membranes for immunostaining with a monoclonal antibody (M1). The peak eluting at 26.8 min in ~44% acetonitrile contained a 31-kDa heparin-binding protein from both seminal fluid and sperm that was recognized by M1 and corresponded to FAA. Position of molecular weight standards is shown at right.

The identification of FAA was achieved by performing Western blot analysis of RP-HPLC fractions with the M1 monoclonal antibody. M1 is known to cross-react with multiple heparin-binding proteins including FAA (Bellin et al., 1996, 1998). Immunoblots of the fraction eluting from RP-HPLC at 26.8 min contained only a single band corresponding to FAA at 31-kDa (Fig. 1B; S.f.), demonstrating the absence of immunogenic HBP contaminants. The mAb did not detect any other proteins in that HPLC fraction, although other faint bands were visible on gels stained with coomassie blue. A coomassie blue-stained acrylamide gel depicts the progressive purification of FAA achieved by solid-phase extraction of HBP followed by RP-HPLC (Fig. 2).

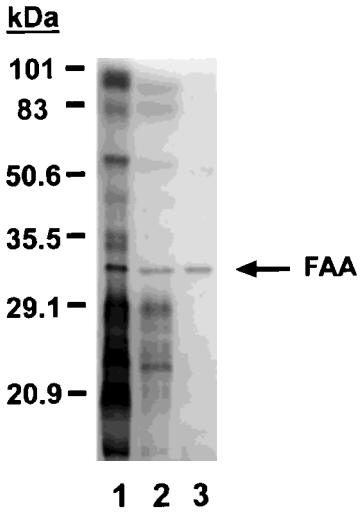

Fig. 2. Sequential purification of fertility-associated antigen (FAA). Electrophoretic characterization of bovine seminal fluid heparin-binding proteins (lane 1) extracted on a solid-phase C4 column (lane 2) and subjected to RP-HPLC (lane 3) demonstrating purification of FAA. Proteins were subjected to SDS-PAGE and stained with coomassie blue. Position of molecular weight standards is shown at left. FAA is indicated by the arrow.

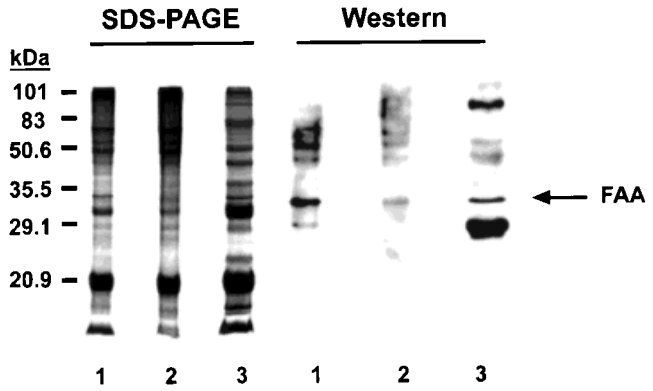

Fig. 3. Western blot of proteins extracted from sperm membranes by hypertonic media. Ejaculated sperm were washed in buffer alone or extracted by incubation with 0.6 N KCl followed by Western blotting. Extracted sperm proteins were solubilized in sample buffer, separated by SDS-PAGE and transferred to PVDF membranes. Proteins reacting with the monoclonal antibody (M1) were detected by enhanced chemiluminescence. A 31-kDa protein corresponding to FAA (indicated by arrow) was detected in immunoblots of whole sperm (lane 1) and in the KCl-extract (lane 3), indicating FAA binds peripherally to sperm cells and is extractable by treatment with hypertonic media. Some residual FAA was detected in sperm after extraction by hypertonic media (lane 2). Position of molecular weight standards is shown at left.

Biochemical Characterization of FAA

Bovine heparin-binding proteins originating from accessory sex glands were previously shown by extraction with hypertonic media, and by Triton X-114 phase separation, to bind to epididymal sperm as peripheral membrane proteins (Miller et al., 1990). We therefore hypothesized that FAA was peripherally bound to sperm membranes and would be susceptible to extraction with hypertonic media. To address the nature of the association between FAA and sperm membranes, ejaculated sperm were extracted with hypertonic PBS (0.6 N KCl) and Western blots were performed on the sperm extract. A 31-kDa protein corresponding to FAA was detected in immunoblots of unextracted sperm and in the sperm KCl extract (Fig. 3; lanes 1 and 3), indicating FAA binds peripherally to sperm membranes and is readily extractable by treatment with high ionic strength media. Supernatants from control sperm preparations incubated for 60 min in PBS contained no immunoreactive protein (data not shown). Although some FAA remained in the salt-extracted sperm samples (lane 2), the amount of FAA present was greatly diminished. Other proteins cross-reacting with M1 in Figure 3 are to be expected as no HPLC purification was performed on the proteins immunoblotted in this experiment.

To confirm that the 31-kDa protein detected in sperm KCl extracts was identical to FAA isolated from seminal fluid, KCl-extracts were analyzed by heparin-affinity chromatography and RP-HPLC. Analysis of sperm membrane HBP by RP-HPLC resulted in a peak eluting at ~26 min (Fig. 1A, insert), similar to elution profiles of seminal fluid-derived FAA (Fig. 1A). That peak contained a 31-kDa HBP that was recognized by M1 by Western analysis (Fig. 1B). Those data indicated that the 31-kDa protein recognized by M1 in KCl sperm extracts was identical to FAA that was purified from seminal fluid and microsequenced. Those data also suggest that FAA is not modified upon binding to sperm membranes.

To determine if FAA consisted of multiple isoelectric variants, HPLC fractions containing FAA were pooled, subjected to 2-D gel electrophoresis, and stained with coomassie blue (Fig. 4). Those data demonstrated that FAA migrated as a basic protein with an isoelectric point ranging from ~7.5 to 8.0. Lower molecular weight proteins (<20 kDa) apparent in Figure 4 represent unknown protein(s) present in the partially purified preparation of FAA presented here. Those proteins did not react with M1 in one-dimensional immunoblots performed prior to 2-D gel analysis (data not shown). FAA migrated as a heterogeneous band, not as a discrete spot, indicating the possible existence of multiple charge variants of FAA. However, analysis of FAA bound to PVDF membranes indicated it was not glycosylated (Fig. 5), suggesting multiple isoforms seen by 2-D electrophoresis were not due to post-translational glycosylation. Although glycosylated proteins were clearly discernible across the spectrum of HPLC fractions presented in Figure 5, FAA (lane 7) was not among those proteins containing carbohydrate residues.

Amino Acid Sequence Analysis

Alignment of the N-terminal amino acid sequence of intact FAA and the amino acid sequences of two peptides derived from enzymatic cleavage of FAA, with a human deoxyribonuclease I-like protein (DNase I-like), is shown in Figure 6. The N-terminal amino acid

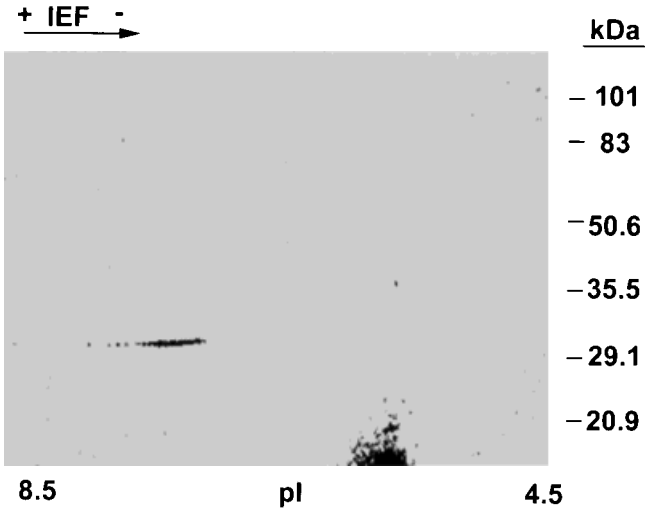

Fig. 4. Two-dimensional gel electrophoresis of fertility-associated antigen (FAA). Seminal heparin-binding proteins were fractionated by RP-HPLC and partially purified FAA was subjected to two-dimensional gel electrophoresis and stained with coomassie blue. Arrow at upper left indicates the direction of first dimension isoelectric focusing from the basic (+) to acidic (−) end. FAA migrated as a heterogeneous band at 31 kDa. Position of molecular weight standards is shown at right.

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FAA              1  LKICSFNVRSFGESKKAGFNAMRVIV 26
                    ||||||||||||||     ||| |||
DNAS1L3         21  MRICSFNVRSFGESKQEDKNAMDVIV 46

FAA Peptide A          QSYLYHDYQAGDADVFSREP
                       || ||||| ||||||||||
DNAS1L3         115    RSYHYHDYQDGDADVFSREP 134

FAA Peptide B          DFVIVPLHTTPEXXV
                       |||| |||||||  |
DNAS1L3         148    DFVIIPLHTTPETSV 159
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Fig. 6. Alignment of fertility-associated antigen (FAA) amino acid sequences with the deduced amino acid sequence of DNAS1L3. Beginning and ending residue number is indicated for known sequences and X represents an undetermined amino acid. Matching residues are indicated by a vertical line. Amino acid sequence comparisons revealed strong identity to a human deoxyribonuclease (DNase) I-like protein (GenBank accession no. U56814). N-terminal sequence of FAA displayed 73% identity to residues 21–46 of human DNase I-like protein. Lys-C digestion of purified FAA and subsequent sequencing of peptide fragments resulted in the identification of two internal amino acid sequences, 15 and 20 residues in length. Peptide A shared 85% identity, and peptide B 80% identity with the human DNase I-like protein.

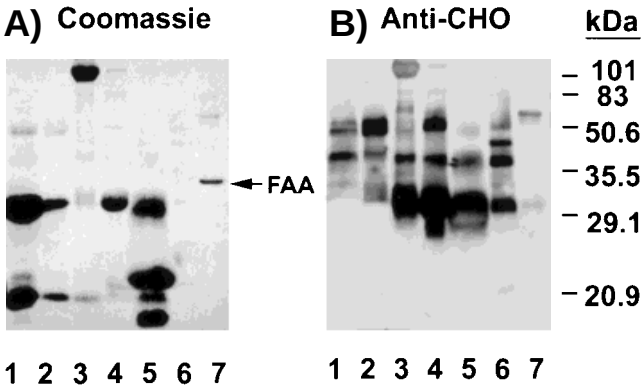

Fig. 5. Glycosylation analysis of seminal heparin-binding proteins and fertility-associated antigen (FAA). RP-HPLC peaks eluting between 10 and 26 min were separated by SDS-PAGE and transferred to a PVDF membrane. (**A**) SDS-PAGE of RP-HPLC fractions after staining with coomassie blue. Lane 1, 10–10.5 min; lane 2, 10.5–11 min; lane 3, 11.5–12 min; lane 4, 16–16.5 min; lane 5, 18–18.5 min; lane 6, 20–20.5 min; lane 7, 26.5–27 min. (**B**) Carbohydrate was detected by biotin-streptavidin labeling as described in Materials and Methods. The 31-kDa FAA in lane 7 (26.8 min peak) was visible by coomassie blue staining (indicated by the arrow) but was not detected by carbohydrate labeling indicating FAA was not glycosylated. Position of molecular weight standards is shown at right.

sequence determined from two separate preparations of purified FAA was LKIXSFNVRSFGESKKAGFNAMRVIV, where X represents an undetermined amino acid. Residue four is likely a cysteine, however, that residue could not be confidently determined. Purified FAA was cored from a polyacrylamide gel and subjected to enzymatic digestion with lys-C to obtain additional internal peptide sequences. Lys-C digestion of purified FAA resulted in the identification of two sequences, peptide A and peptide B, 20 and 13 residues in length, respectively. Those peptides were homologous with the same DNase I-like protein. The N-terminal amino acid sequence of FAA displayed 73% identity, peptide A shared 85% identity, and peptide B shared 80% identity with the DNase I-like protein.

Origin of FAA Secretions

To determine the site of production of FAA, bull accessory sex glands were obtained and heparin-binding protein fractions were analyzed by RP-HPLC. The RP-HPLC peak corresponding to FAA (~26 min elution) was analyzed by SDS-PAGE and immunoblotting with M1. Western analysis indicated the seminal vesicles and prostate gland produced FAA, however, it was barely detectable in tissue extracts from the bulbourethral gland (Fig. 7). Seminal vesicle and prostate FAA differed in appearance on Western blots. Two bands were detected in seminal vesicle extracts, one at ~31 kDa and another at ~33 kDa, while a more diffuse single band at ~33 kDa was detected in prostate extracts.

DISCUSSION

This study identified a fertility-associated antigen (FAA; Bellin et al., 1998) from bovine semen as being homologous to a DNase I-like protein. Three peptide sequences obtained from purified FAA showed high homology with the deduced amino acid sequence from a cDNA (DNase I-like) that shared identity with human DNase I (Rodriguez et al., 1997). No biological function of the DNase I-like proteins has been described al-

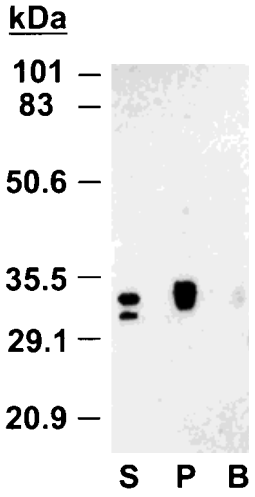

Fig. 7. Western analysis of accessory sex gland extracts. Seminal vesicle (S), prostate (P), and bulbourethral gland (B) tissue extracts subjected to heparin-affinity chromatography and RP-HPLC (26 min peak) were separated by SDS-PAGE and transferred to PVDF membranes for Western blotting. Proteins reacting with the monoclonal antibody (M1) were detected by enhanced chemiluminescence. Immunoreactive FAA was detected in seminal vesicle and prostate gland extracts but not in the bulbourethral gland. Position of molecular weight standards is shown at left.

though conserved calcium-, catalytic, and DNA-binding residues are indicative of a deoxyribonuclease.

FAA is a 31-kDa seminal fluid heparin-binding protein (HBP) that binds to sperm and has been shown in a series of field trials to be positively related to fertility of bulls (Bellin et al., 1996, 1998). Several lines of evidence support the conclusion that seminal FAA purified and microsequenced in this study is the 31-kDa fertility-associated antigen recognized in sperm extracts by M1. Seminal- and sperm-derived FAA exhibited similar chromatographic behavior, identical mobility in SDS-PAGE, and reactivity with M1 in Western blots. Those data suggest no biochemical modification of FAA took place after binding to sperm and associate a novel DNase I-like protein in semen with differences in fertility of bulls. FAA on sperm membranes was readily extracted by treatment with 0.6 N KCl, in agreement with a previous report indicating a peripheral association between bovine seminal HBP and sperm membranes (Miller et al., 1990). Interestingly, sperm-zona binding was increased by pre-incubation of sperm with a crude 0.6 N KCl protein extract from sperm of fertile men (Jean et al., 1995). Those findings confirmed an involvement of KCl-extractable sperm proteins in gamete interactions in humans.

HBP originating from accessory sex glands (Nass et al., 1990) bind to spermatozoa at ejaculation, and binding of HBP to epididymal sperm was sufficient to mediate capacitation by heparin in vitro, thereby regulating the zona-induced acrosome reaction (Miller et al., 1990). Several bovine seminal plasma proteins have stimulatory effects on sperm capacitation. Among those are four acidic bovine seminal plasma proteins (BSP; Manjunath and Sairam, 1987; Manjunath et al., 1993; Therien et al., 1995, 1997). BSP and HBP share the ability to convey heparin-induced capacitation though species-specific differences exist with regard to the effects of heparin on sperm function (Miller et al., 1990; Therien et al., 1997).

The cDNA for BSP-30-kDa (GenBank AF057133) was recently published (Salois et al., 1999). Comparison of the deduced amino acid sequence of BSP-30-kDa with the FAA peptide sequence confirms that BSP-30-kDa and FAA are distinctly different proteins. FAA is associated with increased fertility of bulls (Bellin et al., 1996, 1998), whereas the association of BSP-30-kDa to bovine fertility has not been demonstrated at this time.

Heparin-binding proteins that share antigenic determinants with the family of BSPs are present in the hamster, rat, pig, and human (Leblond et al., 1993). The primary structure of BSPs includes the consensus sequence of fibronectin type-II (FN-II) domains at the C-terminal end (Calvete et al., 1996), characteristic of the major HBP of bull and stallion seminal plasma, but different from all other known FN-II domains (Plucienniczak et al., 1999). In addition to the FN-II domains, BSPs contain unique N-terminal extensions and display differences in glycosylation. Functionally similar heparin-binding proteins from stallion (Calvete et al., 1995a; Magdaleno et al., 1997) and boar (Calvete et al., 1995b; Varela et al., 1997) seminal plasma have been characterized as predominantly BSP-like or belonging to the spermadhesin family. Spermadhesins are a family of multi-functional seminal vesicle products possessing heparin-binding, zona glycoprotein-binding, and serine protease inhibitor-binding activities (Töpfer-Petersen et al., 1995, 1997).

The identification of FAA as being homologous to a human DNase I-like protein and not the BSPs or spermadhesins was unique given the ubiquitous distribution of those heparin-binding protein families in seminal plasma. That finding also suggests functionally undefined DNase I-like proteins may play a role in modifying fertility potential of bulls. DNase I activity has been characterized in seminal fluid and is a component of human prostatic secretions (Yasuda et al., 1993), as well as rabbit seminal vesicle and epididymis secretions (Takeshita et al., 1994). DNase I is believed to be responsible for apoptotic internucleosomal DNA degradation (Peitsch et al., 1993) and is present in the nuclei of spermatogonia and spermatocytes where it may control the number of germ cells entering meiosis by apoptotic elimination (Stephan et al., 1996). DNase I immunoreactivity was also present in the acrosome of mature spermatozoa indicating a possible role in the post-epididymal life span of sperm cells (Stephan et al., 1996). In contrast, DNase I-like expression has not been previously demonstrated in the male accessory sex glands or on spermatozoa. Non-overlapping tissue expression patterns of the DNase I and DNase I-like genes suggest that they may have non-redundant biological functions (Rodriguez et al., 1997). Four DNase I-like genes displaying tissue-specific expression pat-

terns are known, all of which possess conserved calcium- and DNA-binding domains along with potential sites for a cleaved signal peptide at the N-terminus (Rodriguez et al., 1997). The DNase I-like gene sequence differs from DNase I in that it predicts an unglycosylated protein, in agreement with the lack of glycosylation of FAA reported in this study. Overall homology of FAA with the DNase I-like protein(s), including potential calcium- and DNA-binding domains, awaits determination of the full length cDNA and corresponding amino acid sequence of FAA.

In the present study, immunoreactive protein was detected in RP-HPLC fractions of HBP isolated from seminal vesicles and prostate gland, but was barely detectable in bulbourethral gland extracts. The immunoreactive bands would appear to be FAA based on chromatographic similarity to seminal fluid-derived FAA and reactivity with M1. The small amount of material liberated from bulbourethral gland extracts may explain the inability to detect FAA from that tissue, thus the possibility that FAA is produced by all three accessory sex glands should not be discounted. Alternatively, gland-specific differences in synthesis of FAA may exist. Nass et al. (1990) showed a 31-kDa HBP that bound to epididymal sperm was produced by rat and bull seminal vesicles and bulbourethral gland. RT-PCR of total RNA from individual bovine accessory sex glands, using primers designed based on FAA amino acid sequences, resulted in successful amplification of a partial cDNA (600 bp) from the prostate and seminal vesicles that encompassed the two internal peptide sequences of FAA (unpublished observations). The same amplification was not successful in an initial attempt using a template of total RNA from the bulbourethral gland, favoring the possibility that FAA synthesis may be accessory gland–specific.

The nature of the differences between seminal vesicle and prostate immunoblot results is not clear at this time. The identification of a ~33-kDa protein, in addition to the 31-kDa band indicative of FAA purified from seminal fluid, could be the result of post-translational modifications or incomplete protein processing. Chemical analysis of seminal FAA demonstrated that it was not glycosylated, indicating that post-translational glycosylation does not account for the appearance of a higher molecular weight isoform. In addition, two-dimensional gel electrophoresis of FAA demonstrated possible charge variants but no mass variants. While the lower band recognized by M1 in seminal vesicle extracts migrated at the expected position of FAA, the 33-kDa species detected in seminal vesicle and prostate tissue extracts may represent a precursor form of FAA that undergoes further processing (e.g., signal peptide/proteolytic cleavage) prior to secretion into seminal fluid. Alternatively, because M1 is not FAA-specific it could be recognizing another slightly larger HBP. A 33-kDa protein is not detected in Western analysis of seminal fluid proteins or sperm extracts using M1. Thus, while it cannot be ruled out, it is unlikely that the ~33 kDa protein detected in seminal vesicle and prostate tissue extracts represents a different HBP, distinct from FAA, that happens to display the M1 epitope.

FAA represents a novel secretory product of male accessory sex glands that binds to sperm and serves as a protein marker for fertility of bulls. Studies addressing the relationship between FAA and bull fertility may lead to a better understanding of the functional significance of DNase I-like proteins in reproductive tissues. Identification of seminal heparin-binding proteins at the molecular level should contribute to our understanding of the molecular basis of heparin-induced capacitation and the complex role of seminal fluid in regulating fertility of bulls.

CONCLUSION

In conclusion, bovine seminal vesicle and prostate glands produce a unique heparin-binding fertility-associated antigen that shares significant amino acid sequence homology with a DNase I-like protein. A DNase I-like protein present in seminal fluid and on the sperm surface may play a role in capacitation and/or sperm cell degeneration and thereby modify male fertility potential. Studies are underway to examine the functional relationship between presence of FAA on sperm and increased fertility potential of bulls.

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