

INTERACTION OF BOVINE SEMINAL PROTEINS WITH NEUTROPHILS

by

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DEDICATION

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ABSTRACT

Neutrophils ordinarily infiltrate the female reproductive tract subsequent to mating or artificial insemination, resulting in reduced fertility. Recently, it was demonstrated that equine neutrophil extracellular traps (NETs) entangled sperm in these DNA-rich structures, interfering with their normal transport through the female reproductive tract. Seminal plasma (SP) or proteinaceous extracts from SP inhibited sperm-neutrophil binding and specifically degraded sperm-activated NETs, without suppressing bactericidal activity of neutrophils. Fertility-associated antigen (FAA), a 31 kDa naturally occurring heparin-binding protein (HBP) produced by the accessory sex glands, has been shown to bind to sperm and potentiate heparin-induced capacitation. FAA shares 87% identity with DNase I-like family members, and contains two internal DNase-I-like peptide motifs. The purpose of this study was to determine if a recombinant form of FAA displayed capacitating effects associated with the native protein and to determine whether rFAA displayed DNase activity similar to SP or SP protein extracts to inhibit sperm-neutrophil binding.

CHAPTER 1

LITERATURE REVIEW

Introduction

Seminal plasma (SP) was first believed to be important only as a medium for sperm transport. However, more recent findings have shown that SP contains a vast array of constituents important to sperm maturation and fertility potential. These SP constituents facilitate or inhibit capacitation, fertilization, and related events, thus contributing to male fertility. One particular SP constituent of interest is fertility-associated antigen (FAA). This 31-kDa heparin-binding protein found on sperm membranes has been correlated to increased fertility among bulls, but its mode of action still remains unknown. Two apparent functions of FAA are its involvement in heparin-induced capacitation of bovine sperm and its DNase I-like properties which may play a role in freeing sperm entangled in neutrophil traps in the female reproductive tract. This would enable progression up the female reproductive tract to the site of fertilization.

Capacitation

Ejaculated sperm are not capable of penetrating an oocyte until they undergo two fundamental processes, capacitation and acrosome reaction. Capacitation physiologically occurs when spermatozoa reside in the female reproductive tract for a minimum period of time. All spermatozoa do not capacitate at the same rate of time, but rather capacitate heterogeneously over a relatively long period of time reflecting

individual sperm differences and location within the tract. Capacitation also varies among species. Some species produce spermatozoa which require a period of time that allows the acrosome to react (mammals) while other species (birds, sea urchins, other invertebrates) produce spermatozoa that immediately undergo acrosomal exocytosis. Capacitation can also be induced *in vitro* under the appropriate conditions (Yanagimachi, 1994). During this time, spermatozoa undergo a series of biochemical and biophysical modifications that render the ejaculated sperm competent of fertilizing the oocyte. These biochemical changes are associated with modifications in sperm surface protein distribution, alterations in plasma membrane characteristics, changes in enzymatic activities and modulation of expression of intracellular constituents (Yanagimachi, 1994).

One of the better characterized biochemical events during capacitation is the modification of intracellular concentration of calcium ions (Ca^{2+}). Several studies in various mammalian species have demonstrated an increase in Ca^{2+} concentrations during capacitation (Coronel et al., 1987; DasGupta et al., 1993; Fraser and McDermott, 1992; Okamura et al., 1993; Suarez et al., 1993; White and Aitken, 1989; Zhou et al., 1990). Both extracellular and intracellular Ca^{2+} are required for sperm capacitation to occur *in vitro* (DasGupta et al., 1993; Fraser, 1987; Luconi et al., 1996; Roldan and Fleming, 1989; Stock and Fraser, 1989; Visconti et al., 1995). Fraser and McDermott reported in 1992 that spermatozoa intracellular Ca^{2+} was regulated by the Ca^{2+} -ATPase which acts as a Ca^{2+} extrusion pump. Later in 1995, Fraser published his findings that the intracellular Ca^{2+} is also regulated by a $\text{Ca}^{2+}/\text{H}^{+}$ exchanger system with $\text{Na}^{+}/\text{Ca}^{2+}$

antiporter which acts as the Ca^{2+} entrance system in the plasma membrane of spermatozoa. Other researchers also published their findings that intracellular Ca^{2+} stores may possibly act as the intracellular regulators (Blackmore, 1992; Serres et al., 1994). In addition to Ca^{2+} , intracellular ion concentrations of K^+ (Zeng et al., 1995), Na^+ (Hyne et al., 1985), and Cl^- (Faser, 1995) have been shown to be altered during the capacitation process.

Changes in the distribution and composition of plasma membrane lipids and phospholipids are another important feature of sperm capacitation (Baldi et al., 1996). Yanagimachi (1994) linked these changes in membrane lipids and phospholipids to an increase in membrane fluidity. The removal of cholesterol is among these changes and leads to a decrease in the cholesterol: phospholipid molar ratio in the sperm plasma membrane (Benoff, 1993; Ehrenwald et al., 1988, 1990; Go and Wold, 1983, 1985; Hoshi et al., 1990; Langlais and Roberts, 1985). An increase in phospholipid methylation and increased synthesis of phosphatidylcholine from phosphatidyl-ethanolamine has also been associated with capacitation of sperm in vitro (Llanos and Meizel, 1983).

Acrosome Reaction

The final step of capacitation includes the acrosome reaction. The acrosome is a Golgi-derived, membrane-bound, cap-like structure that covers the anterior one-third to one-half of the sperm nucleus (Yanagimachi, 1994). The anterior cap and a posterior region called the equatorial segment make up the acrosome (Eddy and O'Brien, 1994). Proteolytic enzymes required for penetration of the zona pellucida, the glycoprotein coat

surrounding the ovum, and fusion with the egg plasma membrane, are housed in the acrosome. These enzymes, which are analogous to a lysosome or a zymogen granule, are released by exocytosis in response to the binding of the sperm to the zona pellucida.

Zona pellucida protein-3 (ZP3), a sulfated glycoprotein in the zona pellucida of mammals, is the egg protein that physiologically induces the acrosome reaction (Yanagimachi, 1994). Oviductal, follicular fluid and several proteins in the cumulus oophorus matrix have been reported to stimulate the acrosome reaction *in vitro* (Baldi et al., 1996). Among these *in vitro* molecules that induce acrosome reactions are ZP3 (Leyton and Saling, 1989), serum albumin (Meizel, 1985), epidermal growth factor (Lax et al., 1994), atrial natriuretic peptide (Zamir et al., 1995), platelet-activation factor (Angle et al., 1993), and progesterone (Baldi et al., 1995).

Following the stimulation of spermatozoa with ZP3 and progesterone, receptor aggregation occurs in the sperm plasma membrane (Leyton and Saling, 1989, Tesarik et al., 1992). The receptor aggregation is followed by a cascade of downstream membrane and cytosolic signaling factors involved in the acrosome reaction and include calcium, phospholipases and protein kinases (Baldi et al., 1996).

Calcium plays a role in receptor-mediated response and membrane fusion processes in spermatozoa (Yanagimachi, 1994). The presence of Ca^{2+} channels was first shown in the sperm plasma membrane of sea urchins (Darzon et al., 1988). Florman and colleagues (1989) were able to show a rapid (2-5 min) increase in the amount of intracellular Ca^{2+} following the addition of ZP3. This increase was followed by a plateau phase lasting 10-15 min. Later in 1995, Florman and colleagues published their findings

that the acrosome reaction occurred during the plateau phase of intracellular Ca^{2+} influx. Calcium influx can also be induced with progesterone and follicular fluid which are naturally occurring in the female reproductive tract (Thomas and Meizel, 1989; Blackmore et al., 1990; 1991).

In addition, Ca^{2+} plays a role in the fusion of the sperm membrane. In ram spermatozoa, Ca^{2+} is initially associated with the outer membrane of the acrosome. During the acrosome reaction, Ca^{2+} localizes to the fusion sites between the outer acrosomal membrane and plasma membrane anterior to the equatorial segment, followed by localization in both post acrosomal dense lamina and outer acrosomal membrane under the equatorial segment (Watson et al., 1995). Fluxuations in other ions such as Na^+ (Foresta et al., 1993), Cl^- (Turner et al., 1994), bicarbonate (Sauber and Meizel, 1995), and H^+ (Foresta et al., 1993) have also been reported to occur during the acrosome reaction and may be important to the membrane fusion process.

The Role of Seminal Plasma (SP) in Capacitation

The complex mixture of secretions that make up SP contains proteins, some of which are associated with male fertility. One class of SP proteins is the phosphatidylcholine-binding proteins which are found in bovine seminal fluid. These acidic proteins named PDC-109 (BSP-A1/A2), BSP-A3, and BSP-30-kDa, collectively called BSP proteins, make up the majority fraction of bovine SP and bind to the surface of spermatozoa upon ejaculation (Therien et al., 1995). BSP exhibit both heparin binding (Chandonnet et al., 1990), an important constituent for capacitation, and phospholipid

binding (Manjunath et al., 1994), important for sperm lipid metabolism, therefore potentially playing an important role in the process of capacitation. These BSP have also been reported to play a role in forming an oviductal sperm reservoir by enabling sperm to bind to the oviductal epithelium along with increased sperm motility in bovine oviductal epithelium (Gwathmey et al., 2006).

PSP-1 and PSP-II spermadhesin were isolated and characterized from boar SP (Nimtz et al., 1999). These proteins inhibit the production of polyspermic oocytes fertilized from frozen-thawed sperm (Caballero et al., 2004). Spermadhesins are a novel family of secretory proteins, peripherally associated to the sperm surface, expressed in the male genital tract of pig, horse and bull (Topfer-Petersen et al., 1998). The structure and function of spermadhesins have been thoroughly investigated in the pig. This class of proteins includes AWN, AQN-1, AQN-2, PSP-I and PSP-II which exhibit a range of ligand-binding abilities suggesting they may be involved in various steps of fertilization (Topfer-Petersen et al., 1998).

Heparin-binding proteins (HBP) are another class of proteins found in SP. Within this class is a 31-kDa protein named fertility-associated antigen (FAA) that is produced in the male accessory sex glands and binds to distinct regions of ejaculated bull sperm (McCauley et al., 1999). A second HBP that has been characterized from bovine SP is the 24-kDa tissue inhibitor of metalloproteinases-2 (TIMP-2) (McCauley et al., 2001). Both these HBP have been related to increased fertility in the bull.

Seminal Plasma Proteins Potentiate Heparin-Induced Capacitation

Capacitation of bovine sperm can be induced *in vitro* with heparin or chemically related glycosaminoglycans (GAG). Studies using an *in vitro* capacitation assay have shown that bovine epididymal sperm require 22 h to acrosome react in response to GAG isolated from bovine follicular fluid, while washed ejaculated sperm require only 9 h to acrosome react to the same treatment (Lenz et al., 1982; Handrow et al., 1982). Of particular interest is the fact that epididymal sperm exposed to SP for 20 min and then washed, acrosome react in 9 h, equivalent to that of ejaculated sperm (Lee et al., 1985). Therefore, short-term exposure to SP enhances the capability of spermatozoa to undergo spontaneous acrosome reactions. Similar results have also been obtained when zona pellucida was used to induce acrosome reactions. Ejaculated sperm responded to solubilized zonae pellucidae by acrosome reacting when the spermatozoa were first treated with heparin for 4 h, but epididymal sperm were unable to respond (Florman and First, 1988a). When epididymal sperm were exposed to SP for a short period of time they were also able to respond to the solubilized zona pellucida by undergoing the acrosome reaction. These findings suggest that factors present in SP transmit to epididymal sperm the ability to respond to the capacitating effects of GAG and zonae pellucidae with an increase in acrosome reactions in a manner similar to that of ejaculated sperm.

SP also displays decapacitating capabilities. In 1957, Chang found that capacitated sperm could be returned to the decapacitated state by co-incubation with SP. This finding led to the belief that SP was responsible for the initial stabilized state sperm

must maintain during storage in the male reproductive tract. Rabbit sperm were treated with SP from heterologous species resulting in the discovery of decapacitating factors in SP from bull, human, rhesus monkey, boar, stallion, cat, and guinea pig (Oliphant et al., 1985). These decapacitating factors could be pelleted from SP by high speed centrifugation and were thought to be glycoprotein in nature (Bedford and Chang, 1962). Acrosome stabilizing factor (ASF) was later isolated from rabbit SP and believed to be responsible for the sperm decapacitation factor by preventing acrosome reactions (Eng and Oliphant, 1978).

Heparin-Binding Proteins

Heparin belongs to the heparan sulfate family of GAG. GAG are long unbranched polysaccharides or sulfated sugar chains that play important roles in neuronal communication by binding to different proteins. GAG are highly negatively charged molecules primarily found on the surface of cells or in the extracellular matrix. Solutions containing GAG exhibit high viscosity as a result of their extended conformation. This rigidity provides structural integrity to cells and provides passageways between cells, allowing for cell migration. GAG found in tissue include heparin, heparan sulfate, dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitin sulfate D, chondroitin sulfate E, hyaluronic acid, and keratan sulfate.

Chondroitin sulfate proteoglycans isolated from bovine follicular fluid can induce acrosome reactions in bovine epididymal spermatozoa in 22 h and ejaculated spermatozoa in 9 h (Lenz et al., 1982). Porcine follicular fluid also contains GAG which

induce AR in porcine ejaculated spermatozoa (Reyes et al., 1984). In a test to compare commercially available GAG, heparin (10 µg/ml) from porcine intestinal mucosa was found to be the most potent at inducing acrosome reactions of bovine epididymal spermatozoa (Handrow et al., 1982).

Heparin binds to rabbit, bull, and monkey sperm saturably and reversibly in a pH-, calcium-, and temperature-dependent fashion evenly over the entire sperm surface (Handrow et al., 1984). The binding exhibited by heparin on sperm surfaces is typical of receptor-ligand interactions. Bovine ejaculated sperm have more heparin-binding sites than epididymal sperm and when epididymal sperm are incubated with SP the number of heparin-binding sites increase on the sperm (Lee et al., 1985).

A monoclonal antibody M1, specific for high-affinity HBP complexes 21.5-, 24-, and 31-kDa, was used to localize specific HBP. The localization patterns displayed by the M1 antibody were in the acrosomal and postacrosomal regions with minimal binding to the mid-piece of ejaculated bovine spermatozoa (M^cCauley et al., 1996). These proteins were not detectable in epididymal sperm. When epididymal sperm were incubated with seminal fluid for 20 min, seminal fluid HBP were detected on the sperm membranes. These findings support the belief that HBP present in seminal fluid bind to sperm membranes at ejaculation (M^cCauley et al., 1996).

Heparin-Binding Proteins' Relationship to Fertility

The concentration of total HBP in seminal fluid or on sperm membranes does not differ among bulls (Bellin et al., 1994), and the number of heparin-binding sites per

sperm does not vary among bulls (Marks and Ax, 1985). However, sperm from high-fertility bulls have been shown to produce sperm that undergo significantly greater percentages of AR in response to heparin-like material (Ax et al., 1985; Ax and Lenz, 1987; Lenz et al., 1988) along with a greater binding affinity for heparin (Marks and Ax, 1985) when compared to sperm from lower-fertile bulls.

HBP found in SP forms complexes with five affinities for heparin when isolated by affinity chromatography (Miller et al., 1990; Nass et al., 1990). Sperm membranes possessing heparin binding proteins with the greatest affinity for heparin (HBP-B5) corresponded to those bulls being 17 percentage points higher fertility than groups of bulls with other HBP profiles (Bellin et al., 1994). In an evaluation of 343 range beef bulls of four different breeds, bull fertility potential was able to be determined based upon the identification of HBP on sperm membranes (Bellin et al., 1994). The authors concluded that the presence of specific HBP on sperm indicates affinity of sperm to bind heparin, the corresponding ability of sperm to acrosome react, and thus the fertility potential of a bull.

Fertility-Associated Antigen: A Specific Heparin-Binding Protein

A 31-kDa heparin-binding protein coined fertility-associated antigen (FAA) belongs to the class of high affinity HBP, HBP-B5. FAA was identified in sperm membranes of beef bulls and correlated to higher fertility (Bellin et al., 1998). FAA was first identified by the use of a M1 monoclonal antibody produced against seminal fluid HBP-B5 from a vasectomized bull (Bellin et al., 1996).

The site of FAA production was analyzed in heparin-binding protein fractions from bull accessory sex glands and isolated using reversed-phase high performance liquid chromatography (RP-HPLC). The RP-HPLC peak corresponding to FAA was analyzed by SDS-PAGE and immunoblotting with the M1 monoclonal antibody. Western analysis indicated the seminal vesicles and prostate gland produced FAA, while detection in the bulbourethral gland was minimal (M^cCauley et al., 1999).

Fertility-Associated Antigen: Relationship to Fertility

Breeding soundness evaluations (BSE) are commonly performed to evaluate breeding potential of range bulls. BSE is a combination of measurements that include scrotal circumference and physical semen characteristics that can identify bulls as potential satisfactory breeders (Chenoweth et al., 1992). This evaluation, however, cannot identify subfertile bulls (unless they fail the test) or predict fertility potential of individual bulls.

FAA has been identified as a marker for male fertility and in combination with the BSE can improve selection of breeding sires. Sperm from bulls with more HBP-B5, which includes the FAA protein, bound to sperm membranes exhibit higher fertility when compared to bulls with less HBP-B5 on sperm membranes (Bellin et al., 1994). The presence of FAA and two other HBP (21.5-kDa and 24-kDa), collectively known as HBP-B5, in sperm membranes from bulls of several breeds of cattle were evaluated in relationship to fertility potential. FAA-positive bulls with high or low serving capacities were more prolific than FAA-negative bulls in the first 40 d of the breeding season

(Bellin et al., 1998). Breeding cows to FAA-positive bulls resulted in a greater percentage of pregnancies and more cows impregnated earlier in the breeding season (Bellin et al., 1998). When bulls were separated on similar physical characteristics by HBP content of sperm membranes a difference in fertility of as much as 40 percentage points was observed (Bellin et al., 1996).

These studies indicate that fertility potential of bulls can be predicted by identifying specific HBP on sperm membranes and provide a superior test for predicting fertility potential of individual bulls. The ability of accessory glands to produce HBP and sperm to bind HBP differ among males; however, the presence of 21.5-, 24-, and 31-kDa (FAA) HBP in sperm membranes is an important determination of fertility potential of bulls (Bellin et al., 1996).

Fertility-Associated Antigen Biochemistry

Purification of seminal FAA was first accomplished with a combination of heparin-affinity, solid-phase extraction, and RP-HPLC (M^cCauley et al., 1999). Isolation from seminal fluid produced by a vasectomized bull resulted in 20 mg of HBP per ml of seminal fluid with ~0.4% of the total protein extraction being identified as FAA. The identification of FAA was achieved by performing Western blot analysis of the RP-HPLC fractions with the M1 monoclonal antibody that recognizes the high-affinity HBP in SP including 31-kDa FAA (Bellin et al., 1996, 1998).

FAA is also readily extractable by treatment with high ionic strength media from sperm membranes. Sperm membranes and FAA were extracted from ejaculated sperm

with a hypertonic saline (0.6 N KCl) followed by Western blots on the sperm extracts. A 31-kDa protein corresponding to FAA was detected in immunoblots of unextracted sperm and sperm KCl extracts indicating that FAA binds peripherally to the membranes (M^cCauley et al., 1999). Heparin-affinity chromatography and RP-HPLC were used to confirm the 31-kDa protein detected in the sperm KCl extracts was identical to FAA isolated from seminal fluid. FAA is not proteolytically cleaved into smaller variants upon binding to sperm membranes because the 31-kDa protein recognized by M1 in KCl sperm extracts was identical in molecular mass to FAA purified from seminal fluid.

FAA was also evaluated for multiple isoelectric variants. Pooled HPLC fractions containing FAA were subjected to 2-D gel electrophoresis and stained with Coomassie blue. The results demonstrated that FAA migrated as a basic protein with an isoelectric point ranging from ~7.5 to 8.0 (M^cCauley et al., 1999). Analysis of seminal-derived FAA bound to PVDF membranes indicated that this protein was not glycosylated based on Western blots with antibodies to various carbohydrates.

Motifs Found in FAA

Purified FAA subjected to lys-C digestion resulted in the acquisition of two internal peptide amino acid sequences. These two sequences along with the alignment of the N-terminal amino acid sequence of intact FAA were analyzed. The N-terminal amino acid sequence of FAA was homologous to a human DNase I-like-III (DNaseIL3), a gene cloned from human liver expressed sequence tag (EST [Rodriguez et al., 1997]) that was

homologous to DNase I, with its deduced amino acid sequence displaying 87% identity (M^cCauley et al., 1999).

Peptide motifs found in FAA include a protein kinase C phosphorylation site, Casein kinase II phosphorylation site, N-myristoylation site, and cAMP- and cGMP-dependent protein kinase phosphorylation sites. An increase in protein tyrosine phosphorylation has been correlated with sperm capacitation in the mouse, human, and bovine (Visconti et al., 1995). Protein tyrosine phosphorylation mediates a variety of cellular functions such as growth regulation, cell cycle control, cytoskeleton assembly, ionic current regulation, and receptor regulation (Hunter, 1996; Clark et al., 1994). The increase in protein tyrosine phosphorylation is dependent on the presence of BSA, Ca²⁺, and NaHCO₃ in the medium, and the concentrations of those compounds needed for phosphorylation are correlated with those needed for capacitation (Visconti et al., 1995). These findings link phosphorylation to the cascade of events that take place leading up to capacitation of spermatozoa; therefore, the phosphorylation motifs found in FAA may be related to the process of capacitation. Experiments are needed to determine if FAA does cause protein phosphorylation, contributing to downstream signaling to promote capacitation.

DNase I Domain Occurs Twice in FAA

FAA is a member of the family of DNase I-like proteins. The DNase I-like family members differ from DNase I with respect to enzyme activity, regulation, and loci of expression, and their biological role has yet to be defined.

FAA contains two separate DNase I-like motifs. Isolated whole seminal plasma protein extracts have been shown to display enzymatic activity similar to DNase I and play a role in freeing sperm from neutrophils in the female reproductive tract (Alghamdi & Foster, 2005). Neutrophils are released into the female reproductive tract in response to breeding or insemination and have been shown to extrude their DNA causing sperm to become entangled, reducing motility and fertility (Alghamdi et al., 2004). As described above, FAA has been associated with increased fertility in bovine, but its mode of action is still unknown. The presence of two DNase I-like motifs within the FAA sequence suggests that FAA may play an important role in reducing sperm-neutrophil binding in the female reproductive tract leading to its association with increased fertility.

DNase in Semen

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 vesicular and epididymal secretions (Takeshita et al., 1994). DNase I-specific gene transcripts have also been identified in the rat testis and believed to be involved in apoptotic elimination of spermatogonia and spermatocytes by controlling the number of germ cells entering meiosis (Stephan et al., 1996). DNase I immunoreactivity is present in the acrosome of mature spermatozoa indicating a possible role in the post-epididymal life span of sperm cells (Stephan et al., 1996). DNase I-like expression has been identified in male accessory sex glands and on spermatozoa in the form of FAA, a DNase I-like protein (M^cCauley et al., 1999). Spermatozoa can take up naked DNA present in the surrounding

medium (Brackett et al., 1971); therefore, historically the presence of DNase in SP has been suggested to be a defense mechanism to prevent incorporation of foreign DNA into spermatozoa (Carballada and Esponda, 2001). DNase from SP is now believed to play a role in reduction of sperm-neutrophil binding in the female reproductive tract (Alghamdi & Foster, 2005). That phenomenon is described in detail below.

DNase I and II activity has been studied in the oocyte. It is believed that DNase activities in avian oocytes play a role in the degradation of accessory spermatozoa during polyspermic fertilization (Stepinska and Olszanka, 2003). In the mouse, DNase I is believed to be important in sperm decondensation related to the dramatic conformational changes that the chromatin undergoes during fertilization (Bizzaro et al., 2000). These findings suggest DNase is an important factor for both male and female fertility.

Role of DNase Activity in Uterine Biology

Insemination results in the transmission of seminal factors that act, in the female reproductive tract, to promote sperm survival, to condition the female immune response to tolerate the conceptus, and to organize molecular and cellular changes in the endometrium to facilitate embryo development and implantation (Robertson, 2005). The most immediate effect is a rapid and dramatic influx of inflammatory cells into the site of semen deposition. Postbreeding inflammation in the form of increased polymorphonuclear neutrophilic granulocytes (PMN) into the uterine lumen is an important part of the uterine defense mechanism in pigs and horses (Rozeboom et al., 1998; Troedsson et al., 1998). The migration of PMN from the endometrium into the

uterine lumen act to clear the uterus of excess spermatozoa and bacterial contaminants that enter during insemination (Rozeboom et al., 1998; Troedsson et al., 1998).

Several reproductive processes are impacted by the inflammatory response stimulated by SP. Leukocyte recruitment into the endometrial and cervical tissue result in four categories of response functions; (1) the clearance of superfluous sperm and microorganisms introduced into the uterus at mating; (2) the activation of female immune responses specific to paternal transplantation proteins and other antigens present in semen; (3) the tissue remodeling associated with preparation of endometrial receptivity; (4) the activation of the expression of cytokines and growth factors that have been implicated in pre-implantation embryo development (Robertson, 2005). These seminal-plasma-induced changes in the female tissues act to promote survival of spermatozoa and the opportunity for oocyte fertilization, embryo development, and successful implantation (Robertson, 2005).

Phagocytic cells in the cervix and uterus target spermatozoa and bacteria, with selective sperm phagocytosis acting to filter out morphologically abnormal spermatozoa (Tomlinson et al., 1992). The presence of neutrophils in the female reproductive tract at the time of semen deposition has been shown to result in sperm-neutrophil binding that reduces motility and fertility of spermatozoa (Alghamdi et al., 2004).

Neutrophil extracellular traps (NETs) are formed as a result of activated neutrophils extruding their nuclear DNA and associated proteins to ensnare and kill foreign microbes (Brinkmann et al., 2004). Spermatozoa have been shown to activate neutrophils in a similar manner to that of bacteria, leading to the formation of NETs and

extensive sperm entrapment. Sperm-neutrophil binding results in reduced sperm motility. Treating sperm-neutrophil binding with DNase results in reduced binding and improved fertility (Alghamdi & Foster, 2005). The addition of SP was 50% more effective at reducing sperm-neutrophil binding than DNase I treatment alone, suggesting SP constituents other than DNase I are involved in the freeing of entangled sperm cells (Alghamdi & Foster, 2005). It has been suggested that FAA may be involved in dispersing sperm-neutrophil clusters (Alghamdi & Foster, 2005).

In summary, the mechanisms by which sperm capacitate and acrosome react are partially understood. These crucial steps in sperm maturation have been extensively studied in the hunt for molecular mechanism that holds the key to sperm maturation and fertilization potential. Bovine SP contains HBP that interact with sperm and have been suggested to enable oviductal heparin-like GAG to induce capacitation and subsequently the zona-induced acrosome reaction. FAA, a specific HBP found in bovine SP, has been linked to higher fertility among bulls possessing this protein on sperm membranes. FAA's mode of action still remains unclear, but its relationship in heparin-induced capacitation and sperm-neutrophil binding are currently being investigated.

This thesis focuses on the ability of rFAA to function as the native FAA molecule and its involvement in the reduction of sperm-neutrophil binding seen when sperm are treated with SP protein isolates.

CHAPTER 2

EXPERIMENTAL OVERVIEW

Abstract

Fertility-associated antigen (FAA) is a diagnostic indicator of fertility potential among bulls. FAA is produced in the accessory sex glands, binds to sperm in the urogenital tract, and is re-distributed during capacitation. Bovine sperm undergo capacitation in the female reproductive tract; however, this process can be stimulated *in vitro* using defined media. Higher compared to lower fertility bulls produce sperm with a greater propensity to capacitate in a chemically defined medium. Neutrophils are also present in the female reproductive tract in response to insemination. This inflammatory response to sperm and microbial contaminants can be replicated *in vitro* using blood derived neutrophils. Whole seminal plasma has been associated with increased pregnancy rates and litter size and seminal plasma proteins have been shown to reduce sperm-neutrophil binding increasing sperm transport and survival. The objectives of this study were to determine whether FAA, a DNase I-like heparin binding protein, 1) bound to sperm *in vitro* and modulated heparin-induced capacitation and 2) influenced sperm-neutrophil binding of bovine spermatozoa. A 603 bp recombinant bovine FAA (rFAA) was synthesized by PCR from seminal vesicular cDNA and donated by TMI Laboratories International Inc., Tucson, AZ. The donated rFAA was characterized by SDS-PAGE and Western blotting (see Appendix C), and used in a capacitation and neutrophil-sperm binding assay. The addition of rFAA to bovine sperm led to an increase in acrosome reactions which was highly correlated to male fertility in previous research. Ejaculated

sperm were pretreated for 20 min with rFAA (80 or 160 $\mu\text{g/ml}$), washed to remove any unbound protein, incubated 30 min with neutrophils isolated from bovine blood, and evaluated for sperm-neutrophil binding. A dose of 80 $\mu\text{g/ml}$ resulted in a 30.8 % reduction in sperm-neutrophil binding, and the addition of 160 $\mu\text{g/ml}$ led to a 40.3% reduction in sperm-neutrophil binding when compared to control treatment. The higher dose of rFAA was as effective as 400 $\mu\text{g/ml}$ whole seminal plasma proteins in reducing neutrophil-sperm binding. The observed decrease in neutrophil-sperm binding in this study supports an active role in transportation and survival of viable sperm in the female reproductive tract as a new cellular role of FAA contributed at ejaculation.

Introduction

Seminal plasma is made up of fluids produced by the rete testis, epididymis, and accessory sex glands which are added to sperm during ejaculation. The chemical contributions to seminal plasma from these locations vary, and the nature of all individual components is not known (Troedsson et al., 2005). One well characterized group of seminal plasma components secreted by bull accessory sex glands are heparin-binding proteins (HBP). Binding of these HBP and heparin to cauda epididymal sperm *in vitro* stimulated increased frequencies of zonae pellucidae-induced acrosome reactions (Miller et al., 1990). Seminal fluid derived HBP have been divided into five classes with progressively higher affinity for heparin. HBP complexes in these classes range 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). The 31-kDa HBP has been

identified as fertility-associated antigen (FAA) (Bellin et al., 1998) and was associated with increased fertility potential of bulls (Bellin et al., 1996). FAA is produced in bovine seminal vesicles and prostate glands and is homologous to a DNase I-like protein (M^cCauley et al., 1999).

Seminal plasma has also been shown to contain a protein factor or factors that reduce neutrophil binding to spermatozoa *in vitro* in a dose-dependent fashion and may result in an increased number of spermatozoa reaching the oviduct, leading to an increase in fertility (Alghamdi et al., 2004). Neutrophils are recruited into the female reproductive tract in response to insemination. This inflammatory response is important to the female reproductive tract for the removal of excess spermatozoa and microbial contaminants that enter during the breeding process (Hunter, 2003; Kaeoket et al., 2003; Tremellen et al., 1998; Troedsson et al., 2001, 1998; Rozeboom et al., 1998). The presence of neutrophils in the female reproductive tract at the time of insemination has been shown to bind to spermatozoa, forming extensive clusters (Alghamdi et al., 2001; Rozeboom et al., 2001; Alghamdi et al., 2004) resulting in a reduction of motility (Alghamdi et al., 2001) and fertility (Alghamdi et al., 2004; Rozeboom et al., 2000).

Neutrophils exhibit a unique mechanism by which they extrude their nuclear DNA and associated proteins to form neutrophil extracellular traps (NETs) that ensnare and kill foreign objects (Brinkmann et al., 2004). Bacteria (Brinkmann et al., 2004), yeast (Urban et al., 2006), and sperm (Alghamdi et al., 2005) have been shown to activate neutrophils causing a release of nuclear DNA and formation of NETs. The formation of NETs is observed from motile neutrophils, is faster than the apoptosis time-course, is not

accompanied by cytoplasmic markers, and the neutrophils exclude vital dyes; therefore, this process is not a result of cell death (Brinkmann et al., 2004). DNase activity has been found in the seminal plasma of several species (M^cCauley et al., 1999; Yasuda et al., 1993; Shastina et al., 2003; Carballada and Esponda, 2001; Sato et al., 2003) and has been shown to reduce sperm-neutrophil clusters 50% more efficiently than DNase I (Alghamdi et al., 2005). FAA shares some sequence similarity with DNase I (M^cCauley et al., 1999), but its mode of action has remained unclear. The purpose of this study was to evaluate the effect rFAA had on capacitation and reduction of sperm-neutrophil binding.

Materials and Methods

Chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Transformation, expression, purification, and renaturation of rFAA was performed by TMI Laboratories International Inc., in Tucson, Arizona and donated to the University of Arizona for this project. For Materials & Methods on rFAA production and quality control, see Appendix C. The rFAA was constructed with a C-terminal polyhistidine-tag (His₆-tag) incorporated onto the C-terminal end of the expression protein for the use of detecting and purifying the recombinant protein.

Validation of rFAA Function by Heparin-induced Capacitation

Purified recombinant FAA protein was added to semen samples incubated under capacitating conditions to determine if rFAA potentiated heparin-induced capacitation in the same manner HBP or purified FAA from seminal fluid had shown to display in previous studies (Bellin, personal communication). Cryoperserved semen from 4 individual bulls were thawed in a 38°C water bath for 15 sec and washed 3 X in 1 ml TALP (Tryrode's, albumin, lactate, pyruvate) medium (100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 0.3 mM NaH₂PO₄, 21.6 mM Na lactate, 2 mM CaCl₂, 0.4 mM MgCl₂, 10 mM Hepes, 1 mM pyruvate, 6 mg/ml BSA, 50µg/ml gentamycin, pH 7.4). Washed sperm were incubated with increasing concentration of purified rFAA (0, 6.25, 12.5, 25, 50, 100, or 200 µg/ml in TALP medium) in the presence of 10 µg/ml heparin (sodium salt from porcine intestinal mucosa, Scientific Protein Laboratories, Waunakee, WI) at 38°C for 4 h to induce capacitation (Parrish et al., 1988). Bacterial cell lysate (200 µg/ml) from cells transformed with empty vector served as a negative control.

Following the 4 h incubation, 100 µg/ml of fusogenic agent lysophosphatidylcholine (LPC) was added to induce acrosome reactions in capacitated sperm. One sample in each dose response assay was incubated with heparin alone for 4 h without addition of LPC to determine the incidence of spontaneous acrosome reactions. Sperm were incubated with LPC for 15 min, centrifuged, the sperm pellet was resuspended in 1 ml PBS and pelleted again by centrifugation. The final sperm pellet was resuspended in 0.5 ml PBS and air-dried onto pre-warmed slides (Esco fluoro slides, Erie Scientific, Portsmouth, NH). Slides were fixed in 95% cold ethanol for 20 min and rinsed followed

by a PBS rinse. Sperm were incubated with 5 $\mu\text{g/ml}$ fluorescein-conjugated PSA (FITC-pisum sativum agglutinin, Vector Laboratories Inc., Burlingame, CA) in the dark for 30 min at 4°C to stain acrosomal contents. Slides were rinsed with ddH₂O, vectashield mounting medium with 1.5 $\mu\text{g/ml}$ DAPI (Vector Laboratories Inc., Burlingame, CA) was added, coverslips were applied and sealed with nail polish. Slides were examined for acrosome reactions with a Leica DM4000M (Leica, Chatsworth, CA) epifluorescent microscope equipped with Nomarski optics at 400X magnification. Unreacted sperm with intact acrosomes were observed as cells with fluorescent staining in the acrosomal cap of the sperm while acrosome reacted sperm were indicated by a fluorescent staining pattern of equatorial banding or no head fluorescence.

Extraction of rFAA from Pre-treated Sperm Membranes

Cryopreserved sperm from 2 bulls were supplemented with 25 $\mu\text{g/ml}$ rFAA or with 25 $\mu\text{g/ml}$ empty vector lysate were thawed and washed 3X with TALP medium to remove extender and unbound proteins. Sperm membranes were lysed with 1 part Laemmli sample buffer (BioRad, Hercules, CA) containing 1% β -Mercaptoethanol (Amresco, Solon, OH) to 1 part sperm membranes. Samples were boiled for 5 min., centrifuged to pellet insoluble protein, and SDS-PAGE (Laemmli, 1970) was performed on the soluble fractions using 12% polyacrylamide gels. Prestained molecular mass markers (precision plus dual color standards, Bio-Rad) were applied to one lane. Proteins from the gel were transferred to a nitrocellulose membrane (Trans-blot [0.2 μm], Bio-Rad) which was probed with anti-His (C-term)-HRP conjugated antibody (Invitrogen,

Carlsbad, CA), diluted 1:5,000 in PBS-T (phosphate buffered saline with 3% Tween-20).

Blots were developed with TMB substrate.

Seminal Plasma Protein Preparation

Proteins were precipitated from pooled bovine SP with ammonium sulfate (33% w/v). A saturated solution of ammonium sulfate was made by adding 530 g/L ammonium sulfate into ddH₂O. Ammonium sulfate was mixed with pooled SP and drop-by-drop to a final concentration of 33%. The SP mixture was incubated at 4°C rocking for 30 min. Following the incubation the mixture was centrifuged at 3,000 x g for 20 min at 4°C to pellet precipitated protein. Precipitated protein was resuspended in PBS containing protease inhibitors 10 μM PMSF (phenylmethanesulfonyl fluoride) and 1 μM pepstatin A (equal to the initial volume of SP). SP proteins were dialyzed using 10 kDa MWCO SnakeSkin dialysis tubing (Pierce, Rockford, IL) against 50X volume of PBS containing PMSF and pepstatin A overnight at 4°C. The dialysis buffer was changed twice more to fresh PBS with PMSF and pepstatin A for 4 h at 4°C. Following dialysis, the misfolded and aggregated proteins were removed by centrifugation. Soluble protein was quantified using a BCA protein assay (Pierce) following manufacturer's instructions. Absorbance was determined with a Biophotometer (Eppendorf, Westbury, NY), concentration was calculated, and purity of the SP protein extract was assessed by SDS-PAGE as described above. Protein samples were aliquoted and stored at -20°C.

Detection of Endonuclease Activity in Seminal Plasma and rFAA

Endonuclease activities of crude SP protein and rFAA were compared to bovine pancreatic DNase I by co-incubation with 1 µg of plasmid DNA, pBR322 (New England Biolabs, Ipswich, MA) at 38°C for 20 min. Inactivation of SP and DNase I was also performed by heating to 70°C for 10 min before the addition of plasmid DNA. Treated DNA was then separated by electrophoresis on a 0.5% agarose gel and stained with ethidium bromide. To determine whether DNase activity varied among bulls, samples of whole SP from 6 different bulls were compared to each other and to controls using 1 µg/ml of plasmid DNA as substrate in each treatment. A 1 kb DNA ladder from New England Biolabs was loaded in one lane following manufacturer's recommendations.

To determine whether seminal DNase activity could be enhanced by rFAA, rFAA was added to whole SP of bulls with lower endonuclease activity at concentrations of 160, 80, or 40 µg/ml, incubated at 38°C for 20 min, and separated by electrophoresis as described above.

Preparation of PMNs for Neutrophil-Sperm Binding Assay

Blood was collected from a healthy heifer, and PMNs were isolated with Ficoll-Paque PREMIUM (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) following manufacturer's instructions. Whole blood was collected using heparinized blood tubes. Once collected, blood was diluted 1:1 with PBS and carefully layered over Ficoll-Paque PREMIUM layer at a ratio of 4 ml diluted whole blood: 3 ml Ficoll-Paque PREMIUM. Mixture was centrifuged at 400 x g for 30 min room temperature. Upper layers were

removed leaving the bottom layer containing the red blood cells (RBC) undisturbed. The RBC pellet was resuspended in 5 ml sterile ddH₂O for 45 sec to lyse RBC followed by the addition of PBS up to 50 ml. Lysed RBC mixture was centrifuged and pellet was resuspended in 1 ml PBS. The 1 ml of cells was layered over new Ficoll-Paque PREMIUM to remove any cell debris and centrifuged for 5 min at 400 x g room temperature. Cell pellet was resuspend in 5 ml PBS, centrifuged, and resuspended in 0.5 ml PBS. Cells were diluted 1:1,000 in PBS, counted using a hemocytometer, neutrophil concentration was calculated and diluted to a final concentration of 14×10^6 . Neutrophils were stored on ice up for up to 3 h.

Smears were made from isolated neutrophils and stained using Wright-Giemsa stain (HEMA 3[®] Stain Set, Fisher Scientifics, Middletown, VA) following manufacturer's instruction to ensure neutrophils were isolated. Neutrophils were spread onto microscope slides, slides were air dried, dipped into provided Hema 3 fixative solution 5 times followed by Hema 3 solutions 1 and 2 each 5 times, slides were rinsed with distilled water, allowed to dry, and examined under oil immersion lens 1000X final magnification on a Nikon Eclipse E400 microscope (Melville, NY).

Equine blood was donated by Al-Marah Arabian Horse Ranch in Tucson, Arizona and neutrophils were isolated in the same manner as bovine neutrophils. Isolated equine neutrophils were used in the sperm-neutrophil binding assay with equine semen to validate the bovine assay based upon the equine culture system designed by Alghamdi and Foster (2005).

Sperm Preparation for Sperm-Neutrophil Binding Assay

Cryopreserved semen samples were thawed in a 38°C water bath for 15 sec and washed 3 X in 1 ml TALP. Washed sperm pellets were resuspended in 1 ml TALP, sperm were diluted 1:1,000 in PBS, counted using a hemocytometer. Concentrations were calculated and sperm samples were adjusted to a concentration of 50×10^6 sperm cells/ml using TALP medium. Sperm cells in 15 ml conical tubes were placed in a 38°C water bath until use.

Fresh semen was collected by electro-ejaculation. Neat semen was centrifuged at 400 x g for 3 min to pellet sperm. Seminal fluid was removed and sperm pellets were washed 3 X in TALP medium. Concentrations were calculated and sperm samples were adjusted to a concentration of 50×10^6 sperm cells/ml using TALP medium. Sperm cells were stored at 38°C until use.

Freshly collect equine semen was donated by Al-Marah Arabian Horse Ranch and prepared in the same manner as bovine semen. The equine sperm preparation was used in the sperm-neutrophil binding assay with isolated equine neutrophils.

Sperm-Neutrophil Binding Assay and Evaluation

The effects of SP proteins and rFAA on sperm binding to blood-derived neutrophils were determined after spermatozoa were first incubated with respective treatments for 30 min at 38°C, followed by co-incubation with neutrophils under the same conditions.

Wet mounts of sperm binding to neutrophils were evaluated by light microscopy and expressed as the proportion of neutrophils that bound to at least one spermatozoon. A drop of the sperm-neutrophil mixture was placed on a glass slide, covered with a cover slip, and the number of sperm bound to neutrophils was determined using a Leica DMLS microscope (Bannockburn, IL) at 400X magnification. A minimum of 200 neutrophil cells were counted per slide. T-tests were performed to compare each treatment to the blank control. Statistical comparisons between treatments were not performed because the intent for this study was to look for changes between DNase I, SP protein, or rFAA from the blank control.

The experiment was repeated twice using donated equine samples to validate the culture system to results previous reported by Alghamdi and Foster (2005).

Results

Heparin-induced capacitation

Cryopreserved semen was treated with rFAA to evaluate its ability to potentiate heparin-induced capacitation of bovine sperm *in vitro* as shown in Figure 1. Data were analyzed by ANOVA. The least squares means and SEM are shown. The experiment was replicated using semen from four individual bulls. Increasing concentrations of rFAA increased the percentage of sperm that displayed acrosome reactions. A dose of 25 $\mu\text{g/ml}$ was the optimal dose of rFAA needed to maximize acrosome reactions. No significant difference was seen between doses of 25 $\mu\text{g/ml}$ up to 200 $\mu\text{g/ml}$ rFAA.

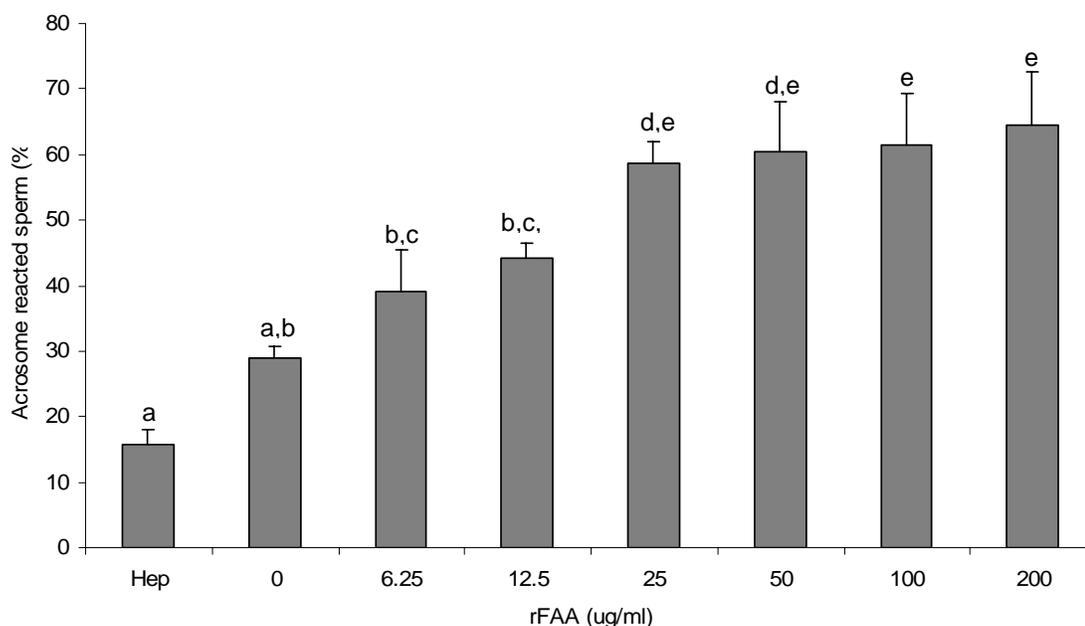


Figure 1. Dose-response induction of acrosome reactions by rFAA. Data were analyzed by ANOVA. Least squares means and SEM are shown. The experiment was replicated using semen from four bulls. Values without a common superscript differ ($P < 0.05$). Increasing concentrations of rFAA were able to increase the percent of bovine sperm that underwent heparin-induced capacitation *in vitro*.

The optimal dose of 25 $\mu\text{g/ml}$ rFAA from the dose-response was used to validate the function of rFAA as a capacitating agent. Following treatment with rFAA or empty vector and induction of acrosome reactions with LPC, sperm were fixed with 95% ethanol to permeabilize sperm membranes. Fixed sperm were incubated with FITC-PSA to stain acrosomal contents and slides were made. Slides were evaluated for intact acrosomes. Sperm with intact acrosomes exhibited fluorescent staining of the acrosome cap while acrosome reacted sperm exhibited staining of the equatorial band or no head fluorescence. Figure 2, below, depicts fluorescent staining on both reacted and intact

acrosomes. The rFAA treated sperm display fewer intact acrosomes when compared to sperm treated with empty vector.

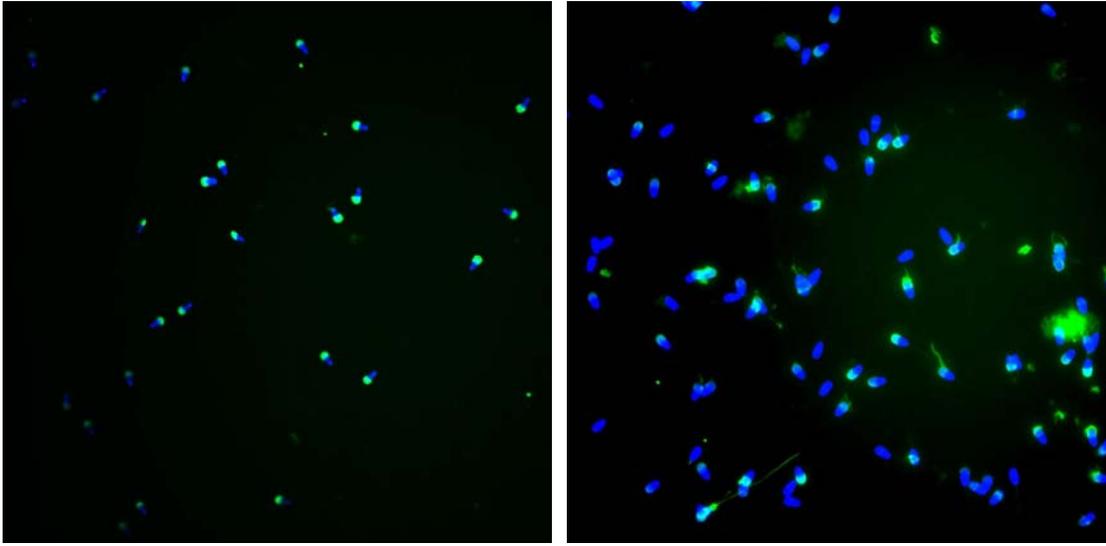


Figure 2. Fluorescent micrograph depicting acrosomal status of bull sperm following treatment with 25 $\mu\text{g}/\text{ml}$ empty vector (left panel) or 25 $\mu\text{g}/\text{ml}$ rFAA (right panel) *in vitro*. Sperm are labeled with FITC-PSA (green) and DAPI (blue). Sperm treated with rFAA displayed fewer intact acrosomes compared to the empty vector treatment.

The data above demonstrating potentiation of capacitation by rFAA in the presence of heparin suggests rFAA is interacting directly with sperm membranes. In order to determine whether rFAA indeed binds to sperm membranes, sperm were co-incubated with rFAA or empty vector, and Western blot analysis was performed on sperm lysates.

Binding of rFAA to sperm membranes was analyzed by treating freshly ejaculated neat semen with 25 $\mu\text{g}/\text{ml}$ FAA or 25 $\mu\text{g}/\text{ml}$ empty vector for 30 min prior to commercial cryopreservation processing. Semen was thawed, sperm were washed to remove any

unbound protein, and cells were lysed and evaluated by Western blotting. Recombinant FAA was detected in samples of sperm cell lysates using an anti-His antibody specific to the C-terminal His_(6X) tag present on the recombinant fusion protein. Sperm treated with empty vector did not react with the anti-His antibody.



Figure 3. Recombinant FAA bound to sperm membranes. Freshly collected neat semen from 2 bulls was supplemented with 25 $\mu\text{g/ml}$ empty vector (lanes 1 and 3) or rFAA (lanes 2 and 4) prior to commercial cryopreservation. Semen was thawed, sperm were washed 3X, and cell lysates were evaluated by Western blotting (anti-His antibody). Treatment of sperm with 25 $\mu\text{g/ml}$ rFAA resulted in binding to membranes and detection by anti-His antibody. The empty vector treatment had no reaction to the antibody.

Detection of Endonuclease Activity in Seminal Plasma and rFAA

Seminal plasma was pooled from 13 bulls and proteins were precipitated with ammonium sulfate. Precipitated proteins were resuspended in PBS containing protease inhibitors and dialyzed in 10 kDa dialysis tubing as described in the Materials and

Methods section. Soluble proteins were quantified (13 mg/ml) with molecular masses assessed by SDS-PAGE as shown in Figure 4. Whole SP was compared directly with isolated SP proteins. The process was also repeated with 2 individual bulls.

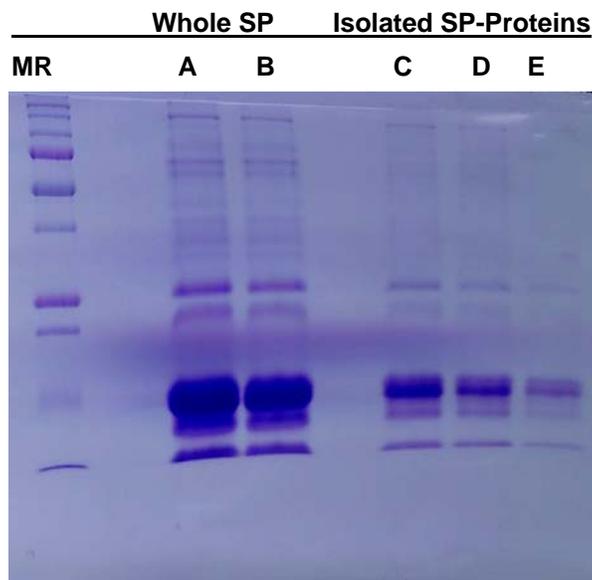


Figure 4. Seminal plasma proteins isolated from pooled bovine SP. Whole SP was diluted to concentrations of A) 4 mg/ml and B) 2 mg/ml with PBS. Isolated SP proteins were diluted to concentrations of C) 2.6 mg/ml, D) 1.3 mg/ml and E) 0.65 mg/ml with PBS. Samples were loaded (10 μ l) and separated on a SDS-PAGE gel followed by Coomassie staining. Proteins were successfully isolated from whole SP.

Plasmid DNA was digested with whole SP from 6 individual bulls to compare endonuclease activity among bulls. A dilution of whole SP at 1:100 was used from each bull. Samples from all bulls displayed the ability to digest plasma DNA, but at different intensities (Figure 5).

In order to compare DNase activity in seminal plasma on an equal protein basis, as opposed to volume dilutions, DNA was co-incubated with various concentrations of isolated SP extracts. Isolated SP proteins were also able to digest DNA when

concentrations of 800 and 400 $\mu\text{g/ml}$ were used. The lower two concentrations of SP proteins were less effective at degrading plasmid DNA, resulting in nicked DNA, creating open circular and linearized DNA which migrates slower than supercoiled plasmid DNA. Alghamdi and Foster (2005) showed both DNase I and SP protein displayed dose-dependent endonuclease activities that were inhibited by heating at 70°C for 10 min. Combination of DNase I and SP protein exhibited an additive enhancement of DNase activity. The addition of DNase I and SP protein resulted in approximately twice as much enzymatic activity as DNase I alone or SP proteins alone (Alghamdi and Foster, 2005).

Recombinant FAA was also tested for endonuclease activity. At concentrations of 400 and 200 $\mu\text{g/ml}$, rFAA was able to completely degrade DNA. Lower concentrations of rFAA, 100 and 50 $\mu\text{g/ml}$, were less effective at degrading plasmid DNA, resulting in supercoiled DNA (lower band) and nicked DNA, creating open circular DNA (upper band) as shown in Figure 6.

The sample in lane 4 of Figure 5 displayed the lowest ability to degrade DNA. That sample was supplemented with 100, 50, 25, or 12.5 $\mu\text{g/ml}$ of rFAA to determine whether intrinsic DNase activity in SP could be modulated by addition of rFAA. The addition of 100 $\mu\text{g/ml}$ of rFAA improved the ability to degrade DNA, concentrations of 50, 25, and 12.5 $\mu\text{g/ml}$ showed little change from whole SP alone (Figure 7).

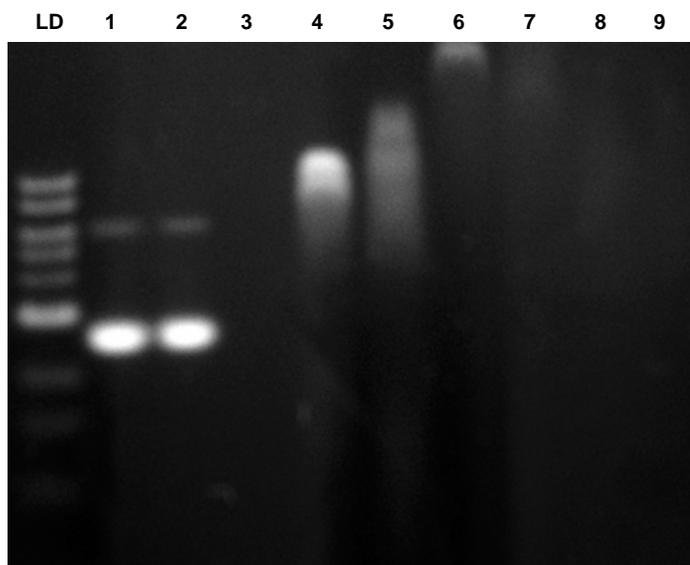


Figure 5. DNase activity of SP from 6 individual bulls. Each lane contained 1 $\mu\text{g}/\text{ml}$ plasmid DNA. Uncut plasmid DNA (lane 1), 400 $\mu\text{g}/\text{ml}$ BSA (lane 2), 0.25 $\mu\text{g}/\text{ml}$ DNase I (lane 3), 1:100 dilution whole bovine SP from 6 bulls (lanes 4-9). LD: 1 kb DNA ladder. The sample in lane 4 displayed the least amount of endonuclease activity and samples in lanes 8 and 9 were able to completely degrade the plasmid DNA.

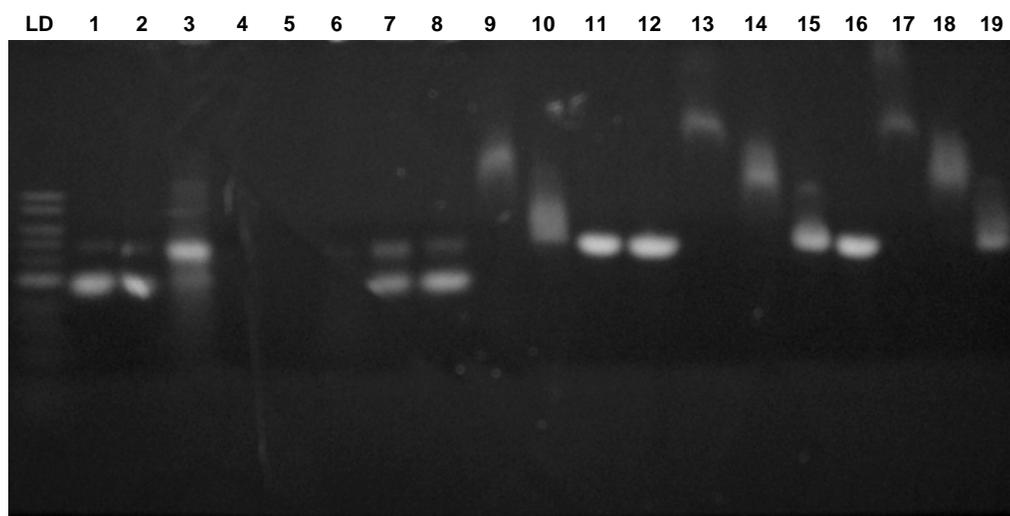


Figure 6. Isolated SP proteins and rFAA endonuclease activity. Each treatment contained 1 $\mu\text{g}/\text{ml}$ plasmid DNA. Uncut DNA (lane 1); 400 $\mu\text{g}/\text{ml}$ BSA (lane 2); 200 $\mu\text{g}/\text{ml}$ empty vector (lane 3); 0.25 $\mu\text{g}/\text{ml}$ DNase I (lane 4); 400 $\mu\text{g}/\text{ml}$ rFAA (lane 5); 200 $\mu\text{g}/\text{ml}$ rFAA

(lane 6); 100 $\mu\text{g/ml}$ rFAA (lane 7); 50 $\mu\text{g/ml}$ rFAA (lane 8); 600 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$, and 100 $\mu\text{g/ml}$ isolated SP from 2 individual bulls (lanes 9-16); 600 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$, and 200 $\mu\text{g/ml}$ isolated pooled SP from 13 bulls (lanes 17-19). LD: 1 kb DNA ladder. Treatment of 400 or 200 $\mu\text{g/ml}$ rFAA was able to completely degrade plasmid DNA. Isolated SP proteins at 600 $\mu\text{g/ml}$ had the greatest ability to degrade plasmid DNA; however, it was less effective than 400 $\mu\text{g/ml}$ rFAA.

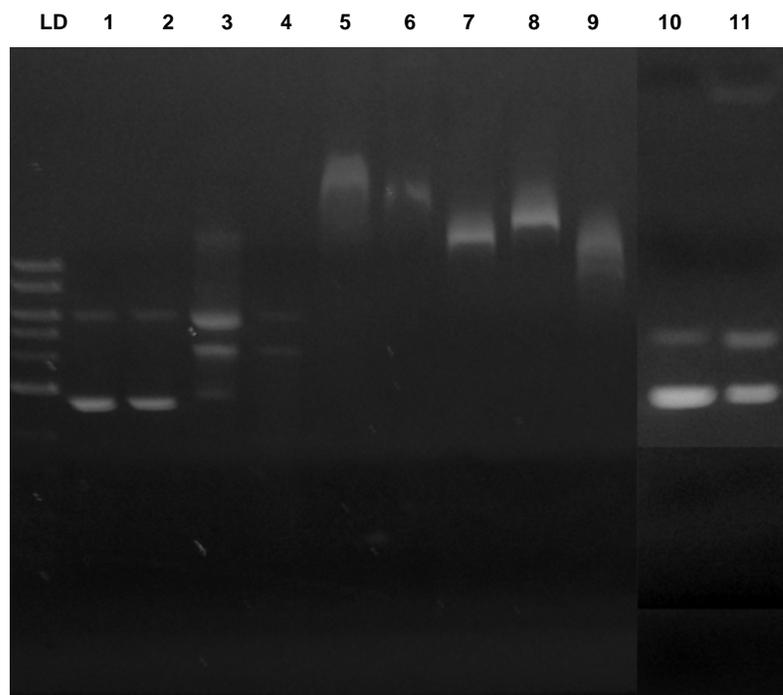


Figure 7. Agarose gel analysis of plasmid DNA treated with whole bovine SP and rFAA. Every lane contained 1 $\mu\text{g/ml}$ plasmid DNA. Untreated plasmid DNA (lane 1), 400 $\mu\text{g/ml}$ BSA (lane 2), 200 $\mu\text{g/ml}$ empty vector (lane 3), 0.25 $\mu\text{g/ml}$ DNase I (lane 4), whole bovine SP diluted 1:100 (lane 5), whole bovine SP with 100 $\mu\text{g/ml}$ rFAA (lane 6), whole bovine SP with 50 $\mu\text{g/ml}$ rFAA (lane 7), whole bovine SP with 25 $\mu\text{g/ml}$ rFAA (lane 8), whole bovine SP with 12.5 $\mu\text{g/ml}$ rFAA (lane 9), heat inactivated DNase I (lane 10), heat inactivated rFAA (lane 11). LD: 1 kb DNA ladder. The addition of 100 $\mu\text{g/ml}$ rFAA to whole SP was able to increase endonuclease activity.

Sperm-Neutrophil Binding Assay

To study the effects of bovine SP proteins and rFAA on the binding of sperm and neutrophils, sperm were pretreated with 160 $\mu\text{g/ml}$ empty vector (negative control), 0.25 $\mu\text{g/ml}$ DNase I (positive control), isolated SP proteins (200 or 400 $\mu\text{g/ml}$), or rFAA (80 or 160 $\mu\text{g/ml}$) and incubated with freshly isolated bovine neutrophils collected from a healthy heifer. The empty vector treatment had no effect on the number of neutrophils bound to sperm when compared to the blank treatment. The DNase I treated sperm exhibited 22 percent less binding of neutrophils to sperm. Treatment with SP proteins and rFAA also reduced the number of neutrophils that bound sperm. The highest dose of SP protein reduced binding by 35 percent and 160 $\mu\text{g/ml}$ rFAA reduced binding by 40 percent when compared to the blank control (Figure 10).



Figure 8. Isolated bovine neutrophil stained with Wright-Giemsa. Freshly isolated bovine neutrophil from blood. Neutrophil extruding DNA forming a NET.

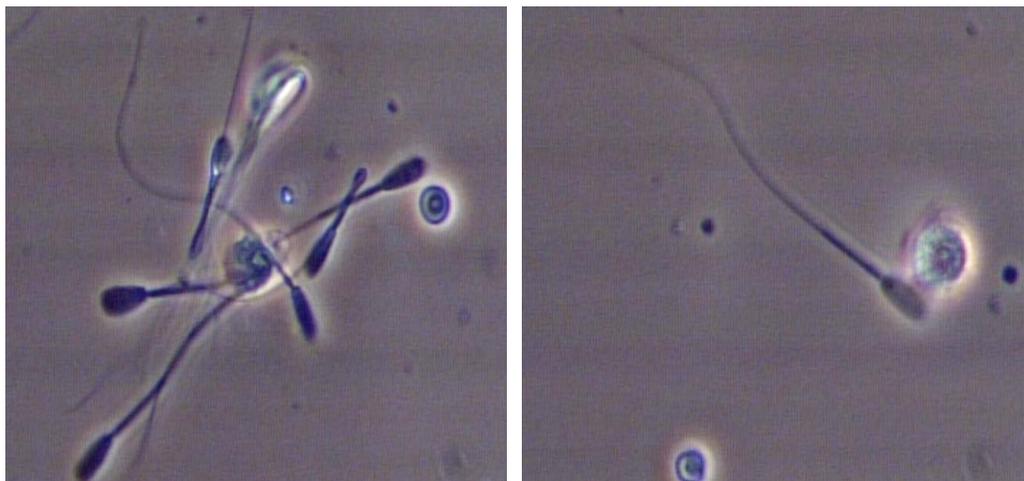


Figure 9. Bovine neutrophil bound to bovine sperm. Left panel showing one neutrophil bound to the tails of multiple sperm following 30 min co-incubation. Right panel showing neutrophil bound to a sperm head following 30 min co-incubation.

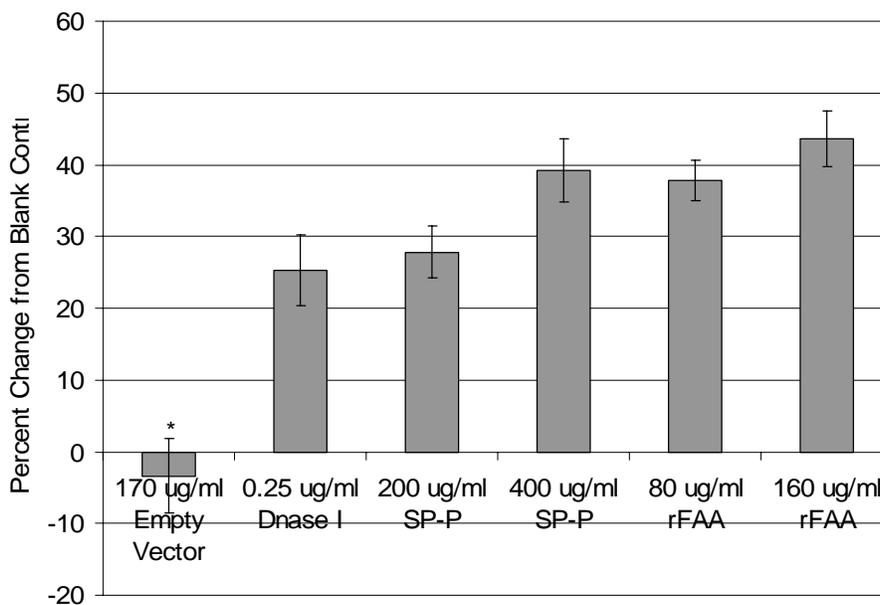


Figure 10. Reduction in sperm-neutrophil binding. Percentage change in sperm-neutrophil binding compared to control. No statistical difference between empty vector treatment and control. DNase I, SP-Proteins (SP-P), and rFAA treatments significantly decreased binding of bovine sperm and neutrophils *in vitro*. The assay was repeated 9 times with 8 individual bulls. *No significant difference was seen between the empty

vector treatment and the blank control. All other treatments displayed a significant reduction in sperm-neutrophil binding when compared to the blank control.

Discussion

Presence of the naturally occurring seminal HBP FAA on sperm surfaces has been linked to higher fertility among bulls. A rFAA was produced as a potential therapeutic additive for cryopreserved semen. The rFAA has been shown to potentiate heparin-induced capacitation *in vitro* (Cropp et al., 2006). This agrees with previous reports showing that HBP or purified FAA from seminal fluid yielded similar results (Bellin, personal communication). All studies showed a dose-dependent relationship to capacitation outcomes in terms of FAA effects on sperm.

The significance of this observation is that the rFAA possesses capacitation-causing activity, so it possesses the function of native FAA found in semen. Also, based upon Dawson (2005), concentrations of FAA detected in bovine seminal fluid were 0-50 $\mu\text{g/ml}$. In this study, the maximum effective dose was 25 $\mu\text{g/ml}$, so it was physiological, not pharmacological in terms of potency (Figure 1).

Few naturally occurring seminal constituents have been identified that enhance capacitation. In addition to FAA, a family of seminal phospholipid-binding proteins known as bovine seminal plasma proteins (BSPs) have been identified and related to sperm function. BSPs have been shown to play an important role in sperm membrane lipid modification events that occur during sperm capacitation (Manjunath & Therien, 2002; Therien et al., 2001; Therien et al., 1997) and play a crucial role in fertilization by

maintaining sperm motility during storage in the female reproductive tract (Gwathmey et al., 2006).

Beyond capacitation effects, this research assigns new unique biological activities to rFAA, specifically, intrinsic DNase activity, and the ability to prevent sperm-neutrophil binding (Figure 6). Those activities were previously ascribed to seminal plasma proteins (Alghamdi & Foster, 2005; Alghamdi et al., 2004). As a positive control, SP proteins, which contain FAA, also demonstrated DNase activity and inhibited sperm-neutrophil binding in this study. The effects of SP proteins were dose-dependent and so were the effects of rFAA.

In previous research, concentrations of 1-8 mg/ml of isolated equine SP proteins were used to inhibit sperm-neutrophil binding. The lowest treatment of 1 mg/ml SP protein resulted in 46% binding and the highest treatment of 8 mg/ml resulted in 15% binding in comparison to 68% binding observed in the control treatment (Alghamdi et al., 2004). In the present study, concentrations of 200 and 400 μ g/ml displayed the same magnitude of reduction in binding of neutrophils to sperm and were able to degrade 1 μ g DNA after a 20 min co-incubation as the previous study by Alghamdi and Foster (2005).

Both freshly collected and cryopreserved bovine semen were tested in the sperm-neutrophil binding culture. There were no differences observed in binding or the reduction of binding between the two types of semen. Overall, the percentage of bovine neutrophils bound to sperm was lower than seen in previous reports conducted with equine samples. Freshly collected equine semen and blood samples were donated by Al-Marah Arabian Horse Ranch in Tucson, Arizona to compare sperm-neutrophil binding to

previous reports. The same neutrophil isolation, sperm preparation, and culture system was used for equine samples as was used for bovine samples. Binding percentages of equine samples were consistent (results not shown) with those previous equine reports (Alghamdi and Foster, 2005). This validated that the bovine culture system was comparable to the equine model, but bovine neutrophils display overall lower binding to sperm *in vitro* under the conditions used in the current study.

Recombinant FAA was able to potentiate heparin-induced capacitation at a concentration of 25 $\mu\text{g/ml}$; however, concentrations of 80 and 160 $\mu\text{g/ml}$ were used to reduce neutrophil binding *in vitro*. These concentrations were slightly higher than observed physiological levels of FAA found in bovine seminal fluid (Dawson, 2004). Higher concentrations of 80 and 160 $\mu\text{g/ml}$ were chosen for the sperm-neutrophil binding assay due to the relative DNase activity displayed by rFAA to degrade plasmid DNA as shown in Figure 6. Sperm were also pretreated with the rFAA and unbound protein was removed before sperm were cultured with neutrophils. Naturally occurring FAA would be present both bound to sperm and unbound in SP and may reduce the concentration necessary to reduce neutrophil binding *in vivo*. The functional activity of the rFAA compared to the native FAA is unknown. The rFAA may exhibit a lower level of activity when compared to the native form, causing a higher than physiological concentration of FAA necessary to inhibit sperm-neutrophil binding. However, sperm treated with a physiological concentration of 20 and 40 $\mu\text{g/ml}$ rFAA would be expected to reduce neutrophil binding by 26 and 32 %, respectively, compared to untreated sperm. Those

values were arrived at by extrapolating a regression line from data actually obtained in these studies.

Comparing the *in vitro* model to *in vivo*, the presence of native FAA would be exposed to neutrophils and NETs for 4 h or longer *in vivo* in the uterus. During this long exposure time, enzyme would continue to have an effect as long as there is substrate available. With increased exposure time and availability of substrate *in vivo* we would expect a greater reduction in neutrophil-sperm binding at lower concentrations compared to the *in vitro* model used in this research. Since capacitation is generally assumed to require 6-8 h, there would be ample time for lower doses of FAA to hydrolyze NETs in the female reproductive tract.

To further investigate the DNase activity of FAA, one possible next step would be to construct rFAA molecules that have one, the other, or both DNase I-like motifs knocked out and constructing a rFAA with the addition of extra DNase I-like motifs to create a possible super agonist. These new recombinants could be used to further investigate the importance of DNase I-like motifs and DNase activity in the function of FAA related to fertility and lead to a concise molecular treatment to improve pregnancy rates by combating the natural influx of neutrophils in the uterus in response to insemination.

Similarly, FAA contains a cAMP phosphorylation site. Phosphorylation of cAMP occurs during the acrosome reaction (Llanos and Meizel, 1983). Therefore, FAA may have several loci within its primary structure that make it an important peptide modulator in terms of fertility. Future experiments will allow molecular dissection of various motifs

within the FAA gene to empirically determine the importance of each loci on FAA's role in increased fertility.

Conclusion

A recombinant seminal protein that naturally occurs in bulls with higher fertility known as fertility-associated antigen (FAA) was produced as a potential semen additive to study its effects on sperm function. The mechanism by which FAA increases bovine fertility still remains unknown and was investigated in this project. FAA, which is a HBP, was added to a 4 h heparin capacitation assay to validate the function of the recombinant protein. Recombinant FAA was able to increase heparin-induced acrosome reactions in a dose dependent manner. This increase in heparin-induced acrosome reactions indicates that FAA may play an important role in the remodeling of the acrosome which is a crucial process that sperm must undergo in order to have the ability to fertilize the ovum.

Fertility-associated antigen is also a DNase I-like molecule, sharing 87% identity with human DNase-I-like-3 (DNaseIL3). The endonuclease activity of rFAA, SP, and isolated SP proteins were assessed by their abilities to digest plasmid DNA. Seminal plasma's ability to digest DNA varied from bull to bull and the addition of rFAA increased the DNase activity of bulls that exhibited lower DNase activity.

Neutrophils were isolated from healthy heifer blood and cultured with sperm to mimic the immune response stimulated in the uterus during the process of insemination. Neutrophils have been shown to release their DNA to form NETs which entangle sperm

reducing their ability to make it to the site of fertilization. Treatment with DNase I, SP protein, and rFAA were able to reduce the number of neutrophils that bound sperm, suggesting that FAA is a major seminal constituent responsible for the NET degradation activity observed in whole seminal plasma.

Collectively these results demonstrate that rFAA potentiates heparin-induced capacitation *in vitro* and displays DNase activity reducing binding of sperm to neutrophils *in vitro*. This reduction may allow sperm to escape the web of DNA extruded by neutrophils in the female reproductive tract and increase the likelihood of sperm reaching the site of fertilization. In conclusion, rFAA exhibited a number of beneficial properties to improve sperm function and helps explain why FAA is a marker for bull fertility. The production of a recombinant FAA protein could be used as a semen additive to increase fertility by increasing capacitation and reducing the number of sperm entrapped in NETs formed in the uterus.

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APPENDIX A

A RECOMBINANT SEMINAL PROTEIN COMBATS INHIBITORY EFFECTS OF NEUTROPHIL EXTRACELLULAR TRAPS (NETs)

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Neutrophils ordinarily infiltrate the female reproductive tract subsequent to mating or artificial insemination, resulting in reduced fertility. Recently, it was demonstrated that equine neutrophil extracellular traps (NETs) ensnared sperm, interfering with their normal transport through the female reproductive tract. A major constituent of NETs was found to be DNA extruded from non-apoptotic neutrophils. Seminal plasma (SP) or proteinaceous extracts from SP inhibited sperm-neutrophil binding, and specifically degraded sperm-activated NETs, without suppressing bactericidal activity of neutrophils. Fertility associated antigen (FAA) is a 31 kDa protein produced in the accessory sex glands which binds to sperm as they traverse the male urogenital tract. FAA shares 87% identity with DNase-I-like family members and contains two internal peptide sequences with conserved DNase-I signature motifs. The purpose of this study was to determine if FAA displayed DNase activity and inhibited sperm-neutrophil binding. To that end, a recombinant bovine FAA (rbFAA) construct spanning 603 bp, including both DNase-I motifs, was cloned from seminal vesicles. The construct, designed to contain a C-terminal (His)₆ tag in a pCR T7/CT-TOPO expression vector, was expressed in *E. coli*. The expressed recombinant (231 aa, 26.6 kDa) was purified by metal affinity chromatography to near homogeneity. Purified rbFAA displayed DNase activity at concentrations of $\geq 3 \mu\text{M}$ as visualized by agarose gel electrophoresis. Sperm from 9 bulls were incubated with various concentrations of rbFAA or SP protein extracts, washed 3X, and sperm were mixed with freshly isolated bovine neutrophils (15×10^6 cells/ml). After 30 min., the proportion of sperm-neutrophil binding was observed microscopically. Whole SP protein extracts at concentrations of 200 or 400 $\mu\text{g/ml}$ inhibited binding by 28 and 40 %, respectively ($P < 0.01$), compared to control. Concentrations of 80 or 160 $\mu\text{g/ml}$ rFAA displayed similar activity to whole seminal plasma extracts, reducing binding by 38 and 44 %, respectively ($P < 0.001$), compared to control. When diluted SP from 6 bulls was incubated with plasmid DNA, degradation indicated that seminal DNase activity varied widely across males. The ability of FAA to inhibit sperm-neutrophil binding provides a potential mechanism to explain the increased fertility observed with FAA-positive bulls. In pigs and horses, co-insemination of extended semen with SP significantly improved pregnancy rates, and litter size also increased in pigs. Whether or not that effect is the result of seminal DNase activity, contributed by FAA, remains to be determined experimentally. (*Submitted abstract to American Society of Andrology, 2007 Annual Meeting.*)

APPENDIX B

RECOMBINANT SEMINAL PROTEINS POTENTIATE CAPACITATION OF BOVINE SPERM IN VITRO

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Type-2 tissue inhibitor of metalloproteinases (TIMP-2) and fertility-associated antigen (FAA) are diagnostic indicators of fertility potential among bulls. They are produced in accessory sex glands, bind to sperm in the urogenital tract, and are re-distributed during capacitation. Bovine sperm undergo capacitation in the female reproductive tract; however, this process can be simulated in vitro using defined media. Higher compared to lower fertility bulls produce sperm with a greater propensity to capacitate in a chemically defined medium. The objective of this study was to determine whether TIMP-2 and FAA, both heparin binding proteins, modulated heparin-induced capacitation of bovine spermatozoa. A 585 bp DNA fragment encoding the entire mature bovine TIMP-2 peptide (rTIMP-2) and a 603 bp FAA amplicon (rFAA) were synthesized by PCR from seminal vesicular cDNA, individually cloned into a pCR T7/CT TOPO vector and expressed as recombinant His-tag fusion proteins. A cobalt-based metal affinity resin was used to purify rTIMP-2 and rFAA from bacterial cell lysates under denaturing conditions. Recombinants were refolded, characterized by SDS-PAGE and Western blotting, and used in capacitation assays. Sperm from replicate ejaculates (n=4 bulls) were incubated in capacitating media supplemented with rTIMP-2 or rFAA (0-200 µg/ml) for 4 h, and acrosome reactions (AR) were induced with lysophosphatidylcholine (LPC). Lysates from cells transformed with empty vector served as negative controls. Following LPC treatment, sperm were stained with FITC-PSA to evaluate acrosome integrity. rTIMP-2 and rFAA independently potentiated heparin-induced capacitation in a dose response manner. Incidence of AR induced by rTIMP-2 and rFAA treatment ranged from 36-57% and 39-64%, respectively. A dose of 25 µg/ml rTIMP-2 resulted in a 1.5-fold increase in AR and addition of 25 µg/ml rFAA led to a 2-fold increase in AR compared to heparin treatment alone. Combined, no synergistic or additive effects were observed. These recombinant isotypes can now be structurally evaluated to determine molecular features that convey capacitation, expanding our knowledge regarding regulation of this complex prerequisite to fertilization. (*Presented at the 39th Annual Society for the Study of Reproduction Meeting, July 29-August 1, 2006, Omaha, NE, Abstract #302.*)

APPENDIX C

PRODUCTION OF RECOMBINANT BOVINE FAA

Materials and Methods

Cloning and Sequencing Analysis

The chemical identity of FAA was first described by the Ax laboratory at the University of Arizona after purification of the native protein from seminal plasma by reversed-phase high performance liquid chromatography (RP-HPLC) and microsequencing analysis (M^cCauley et al., 1999). N-terminal sequence of the intact protein and two internal peptides of lys-C digested FAA were obtained and determined to be homologous with a deduced peptide sequence of a human DNase I-like protein (DNaseIL3; Genbank accession no: U56814). Oligonucleotides were designed using 5' and 3' segments of DNaseIL3 resulting in a 592 bp PCR product transcribed from bovine accessory sex gland RNA. That PCR product was isolated, ligated into pCR[®]2.1-TOPO[®] cloning vector (Invitrogen, Carlsbad, CA) and extended by 5' RACE leading to the identification of a 900 bp cDNA of FAA (disclosed in USPTO 6,891,029). The amino acid sequences previously reported for the native FAA protein (M^cCauley et al., 1999) were embedded in the deduced protein sequence of the FAA cDNA verifying that the authentic cDNA corresponding to native FAA had been cloned.

Bovine FAA cDNA clones (USPTO 6,891,029) were utilized as template to amplify a 603 bp fragment by PCR to be used as an expression clone to produce rFAA. Oligonucleotide primers, forward 5'-atggagaagctaaacggaaat- 3' and reverse 5' –

gctgacatccagggccttc- 3' successfully amplified the 603 bp product of the FAA gene. Cycling conditions used for PCR were: 94°C for 2 min followed by 35 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min; with a 10 min final extension at 72°C on a Mastercycler gradient thermal cycler (Eppendorf, Westbury, NY). The fresh PCR product was directly cloned into the pCR T7/CT-TOPO expression vector using the TOPO[®] TA cloning kit (Invitrogen) following manufacturer's recommendations. Cloned products were transformed using TOP10 F' One Shot[®] chemically competent E. coli (Invitrogen). Transformation reactions were incubated on ice 30 min followed by a heat-shock at 42°C for 30 sec and addition of 250 µl provided SOC medium. Each transformation reaction was placed into an Environ Lab-line shaker (Barnstead International, Dubuque, IA) for 1 h at 37°C at 200 rpm. Aliquots (50 µl) of each transformation were spread onto pre-warmed select Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 1.5% agarose, pH 7.0) plates supplemented with 50 µg/ml ampicillin and incubated overnight at 37°C. Single bacterial colonies from each transformation were selected and inoculated cultured into LB media with ampicillin (50 µg/ml) for an additional 16 h in a shaking (225 rpm) 37°C incubator. Cells were harvested and DNA was purified by miniprep procedures (Qiagen Spin Miniprep columns; Qiagen, Valencia, CA) according to manufacturer's instructions. DNA was air-dried and resuspended in sterile dH₂O at a concentration on 1 µg/ml. Re-PCR was performed using the same oligonucleotide primers described above to confirm presence or absence of the FAA insert in plasmid DNA preparations. Positive clones were selected and sequenced (Applied Biosystemes 373A Automated DNA sequencer utilizing

DyeDeoxy™ terminator chemistry) at the University of Arizona DNA sequencing facility. PCR products were analyzed by agarose gel (2% [wt/vol]) electrophoresis in TBE buffer (90 mM Tris, 90 mM boric acid, 2mM EDTA, pH 8.3) containing ethidium bromide (EtBr; 5 µg/ml) and visualized by ultraviolet illumination. Gels were electrophoresed in a horizontal gel apparatus (Bio-Rad) at 75 V for 20 min followed by 100 V until complete. A 100 bp PCR DNA ladder (EZ Load 100 bp Molecular Ruler, Bio-Rad) served as reference standard. Gel images were analyzed and captured using an ultraviolet light box and CCD camera lined to Alpha Imager™ software (Alpha Innotech Corporation, San Leandro, CA). Nucleotide sequence analyses and comparisons were conducted with GCG software (Version 10.0, Genetics Computer Group, Madison, WI) and The Biology WorkBench version 3.2 software available at <http://workbench.sdsc.edu/>.

Transformation and Expression of rFAA

The rFAA construct (10 ng) was transformed into OneShot BL21(DE3)pLysS cells (Invitrogen) following manufacture's instructions. Cells were mixed with DNA, incubated on ice for 30 min and heat-shocked for 30 sec at 42°C. Provided medium (250 µl SOC) was added and incubated for 30 min at 37°C in a shaking incubator (Innova 4000, New Brunswick Scientific Co. Inc., Edison, NJ). The mixture was then added to 10 ml of LB medium (Luria-Bertani, 1% tryptone, 0.5% yeast extract, 1% NaCl) containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and cells were grown overnight at 37°C with shaking. OneShot BL21(DE3)pLysS cells were transformed with

no DNA (empty vector) to serve as a negative control for protein expression. The overnight culture was inoculated into 500 ml to 1 L of LB medium containing ampicillin and chloramphenicol and grown 2-3 h until they reached an O.D. of approximately 0.6. IPTG (isopropyl β -D-thiogalactoside) was added at a final concentration of 0.5 mM to induce expression of rFAA and cultures were grown for an additional 4 h at 37°C with shaking. Cells were collected by centrifugation (3,000 x g for 20 min at 4°C) and stored at -20°C until purification.

Preliminary experiments were performed to determine the time course of optimal protein expression and whether the recombinant was expressed as a soluble protein or as insoluble material in the form of inclusion bodies. Bacterial cell pellets from 0.5 ml liquid culture were collected at 0, 2, and 4 h following induction with IPTG and extracted in 0.5 ml lysis buffer (50 mM KPO₄, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10mM inidazole, pH 7.8). Samples were resuspended in lysis buffer, frozen on dry ice and thawed at 42°C three times. The insoluble fraction was pelleted by centrifugation at 13,000 x g for one minute and SDS-PAGE (Laemmli, 1970) was performed on the soluble and insoluble fractions with 12% polyacrylamide gels. Prestained molecular mass markers (precision plus dual color standards, Bio-Rad, Hercules, CA) were applied to one lane. Parallel gels were electrophoresed, one was stained with coomassie blue (Brilliant blue R-250) and proteins from the other gel were transferred to a nitrocellulose membrane (Trans-blot [0.2 μ m], Bio-Rad) which was probed with anti-His (C-term)-HRP conjugated antibody (Invitrogen) diluted 1:5,000 in PBS-T (phosphate buffered saline with 3% Tween-20). Detection of the recombinant

was based on the presence of the C-terminal polyhistidine-tag (His₆-tag) incorporated onto the C-terminal end of the expression protein. The blotted membrane was blocked in PBS-T + 5% BSA prior to incubation with antibody. The membrane was rinsed three times in PBS-T + 1% BSA and the blot was developed by incubation in HRP substrate (TMB, Promega, Madison, WI).

Purification of rFAA

The harvested bacterial cell pellet was lysed in 80 ml denaturing extraction buffer (8M urea, 50 mM NaPO₄, 300 mM NaCl, pH 7.0) per liter of culture resulting in a 12.5-fold concentrated extract. The lysed pellet was subjected to three freeze-thaw cycles to ensure complete disruption of cells as described above. The extract was clarified by centrifugation at 13,000 x g and the recombinant was purified using BD Talon immobilized metal affinity chromatography resin (BD BioSciences, Mountain View, CA) according to manufacturer's recommendations. Clarified extracts were mixed with equilibrated Talon resin (8 ml concentrated extract per ml resin) for 20 min, unbound material was removed by centrifugation and the resin was washed 2 X with denaturing extraction buffer. Recombinant protein was eluted from the resin by 250 mM imidazole in extraction buffer. Three bed volumes were collected and pooled after aliquots were taken for electrophoresis. Purity of the recombinant protein was assessed by SDS-PAGE and identity was verified by Western blotting with anti-His antibodies as described above.

Renaturation

Purified rFAA was gradually renatured by a multi-step dialysis using 10 kDA MWCO SnakeSkin dialysis tubing (Pierce, Rockford, IL) against 50X volume of buffer first containing 50 mM NaPO₄, 3 M NaCl, 6 M urea, 0.2 M L-arginine, pH 8.0 at 25°C. After 4 h, the dialysis buffer was changed to a buffer containing 50 mM NaPO₄, 3 M NaCl, 4 M urea, 0.2 M L-arginine, pH 8.5 at 4°C overnight. The dialysis buffer was changed one last time to a buffer containing 50 mM NaPO₄, 0.15 mM NaCl, 2 M urea, pH 7.4 at 4°C for 4 h. Following dialysis the insoluble aggregates of misfolded protein were removed by centrifugation. Soluble protein was quantified using a BCA protein assay (Pierce) following manufacture's instructions. Absorbance was determined with a Biophotometer (Eppendorf, Westbury, NY) and concentration was calculated via non-linear regression multi-point calibration curve using BSA standards prepared in the last dialysis buffer. Protein samples were aliquoted and stored at -20°C.

Results

Transformation and Expression of rFAA

The 603 bp fragment amplified from bovine FAA cDNA clones (USPTO 6,861,029) was used as an expression clone to produce rFAA. The rFAA was transformed into OneShot BL21(DE3)pLysS cells (Invitrogen). Expression of rFAA was induced by 0.5 mM IPTG and samples were collected 0, 2 and 4 h following induction of the protein expression. Extracts were prepared with nondenaturing lysis buffer for the soluble fraction or denaturing 8M urea buffer for the insoluble fraction and analyzed

separately by SDS-PAGE. The insoluble 4 h induced culture exhibited the predominant production of rFAA, see Figure 1.

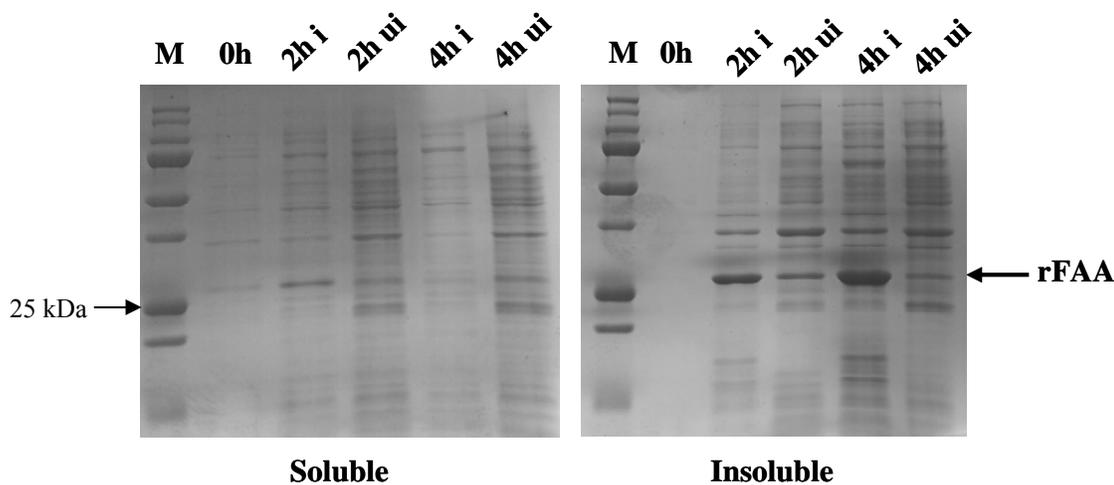


Figure 1. Time course of rFAA induction. Bacterial cultures were induced with IPTG as described in the Materials and Methods. One ml samples were collected at 0, 2 and 4 h relative to induction of protein expression. Induced (i) and uninduced (ui) fractions at each time point are depicted. M: molecular mass marker.

Protein from parallel gels were transferred to a nitrocellulose membrane and probed with anti-His (C-term)-HRP conjugated antibody diluted 1:5,000 as described in the Materials and Methods section. A 26.6 kDa product was detected in the 4 h insoluble induced bacteria fraction corresponding to the predominant band seen in the 4 h insoluble induced bacteria fraction on the coomassie stained gel.

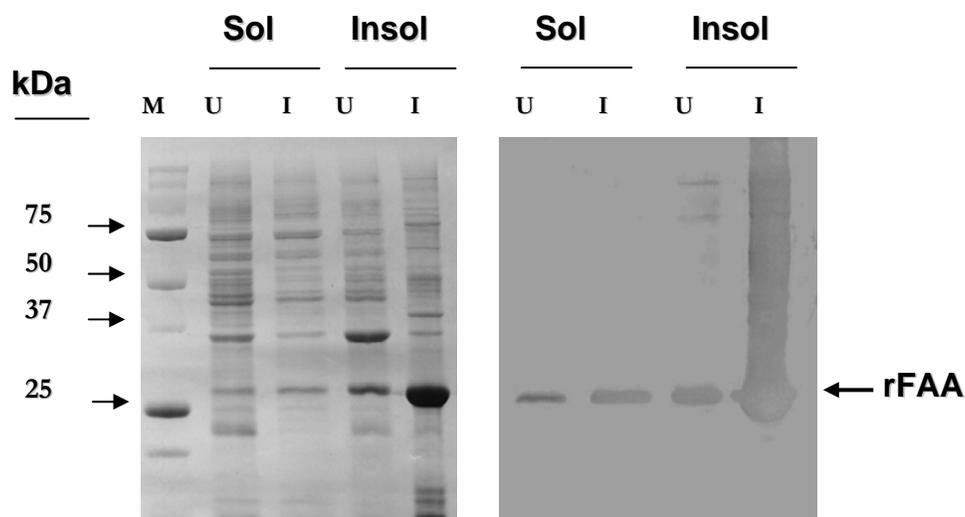


Figure 2. 4 h rFAA induction. Bacterial cultures were induced with IPTG as described in the Materials and Methods. One ml sample was collected at 4 h relative to induction of protein expression. Induced (i) and uninduced (ui) fractions are depicted. M: molecular mass marker. Left: Coomassie stained SDS-PAGE gel. Right: Western blot analysis by anti-His antibody.

Purification of rFAA

Bacterial cell pellets were harvested after 4 h after induction with IPTG and lysed with denaturing extraction buffer. The extracts were clarified and purified using BD Talon immobilized metal affinity chromatography resin. Recombinant protein was eluted with 250 mM imidazole extraction buffer and analyzed by SDS-PAGE and Western blotting with anti-His antibodies. The SDS-PAGE and Western blots exhibited a single 26.6 kDa recombinant protein product, see Figure 3.

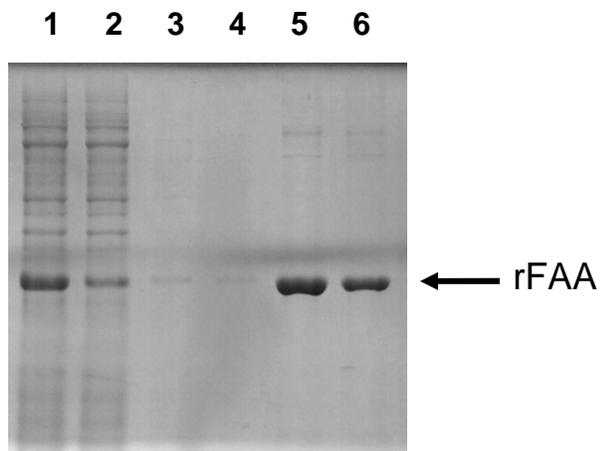


Figure 3. Purification of rFAA. From left to right on the SDS-PAGE gel, first lane, total extract from cultures; second lane, protein not bound to TALON column; third and fourth lanes, column washes; fifth and sixth lanes, protein elution with imidazole.

Renaturation

The purified rFAA was renatured by a multi-step dialysis as described in the previous section. Soluble protein was quantified and resulted in approximately 500 $\mu\text{g/ml}$ to 800 $\mu\text{g/ml}$ varying among bacterial culture batches. Renatured protein was analyzed on SDS-PAGE and Western blot to ensure the protein folded correctly or was not degraded in the renaturing process.

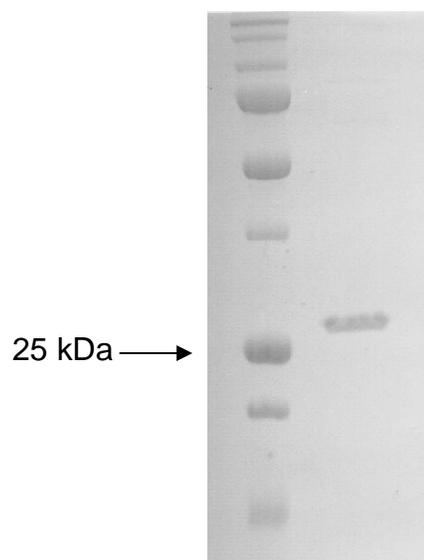


Figure 4. Final refolded and purified rFAA. Western blot analysis of final 26.6 kDa rFAA product.

	<u>Motif 1</u>	<u>Motif 2</u>
Bovine rFAA	151-IVPLHTTPETSVREIDELADV-171	188-GDFNAGCS-195
Human DNase IL3	151-IIPLHTTPETSVKEIDELVEV-171	188-GDFNAGCS-195

Figure 5. Similarity of DNase signature motifs found in bovine rFAA to human DNase-I-like-3 (DNase IL3). Changes in positions 152 and 170 of motif 1 are conserved. Changes in positions 163 and 169 of motif 1 are non-conserved. Bovine rFAA DNase I-like motif 1 shares 90% identity with the motif in human DNase IL3. Motif 2 shares 100% identity between the bovine rFAA and human DNase IL3.