Analysis of Bovine Seminal Plasma Proteins from Korean Native Cattle, Hanwoo, and Korean Native Brindle Cattle

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ABSTRACT

After spermatogenesis, spermatozoa come in contact with fluids in the epididymis where they mature. During ejaculation, spermatozoa are mixed with secretions from prostate gland, vesicular glands, and bulbourethral glands. During natural mating, seminal plasma is deposited in the female reproductive tract eliciting various physiological and immunological responses. With the advances in proteomics, the components of seminal plasma have been identified and the information may be valuable in identifying markers for fertility. Components of seminal plasma that affect fertility have been discovered and the mechanism of action of these factors has been determined. The objective of this study was to determine the specific seminal plasma proteins from Korean native cattle, Hanwoo, and Korean native brindle cattle (KNBC) with the long term goal of improving fertilization rate. After SDS-PAGE and 2-dimensional gel electrophoresis, proteins were identified by Q-ToF analysis. They include plasma serine protease inhibitor precursor and platelet-activating factor acetylhydrolase after SDS-PAGE. Number and density of the spots in 2-dimensional gels were higher in KNBC than Hanwoo. Proteins identified from the paired spots of both breeds include chain A, bull seminal plasma PDC-109 Fibronectin Type II module, BSP-30 kDa precursor, and Spermadhesin Z13 or its precursor. Interestingly, some proteins were identified from multiple spots. The functional differences of these diverse forms of the proteins may require further studies. With their previously reported roles in sperm capacitation by these proteins, the studies on the mechanism of action, ligand interaction and the variation in the genome may help improving fertility in cattle.

(Key words : Seminal plasma protein, Fertilization, Hanwoo, Korean native brindle cattle)

INTRODUCTION

Seminal plasma is produced mainly from the male accessory sex glands and provides the majority of the semen volume. After mating, one of the main functions of the seminal plasma is to provide means of transport for sperm in the female reproductive tract. Components of the seminal plasma which bind sperm are involved in the fertilization process including capacitation. Through the development of artificial insemination, embryo transfer, and *in vitro* fertilization, the importance of seminal plasma in the fertilization process has not been well recognized. With the recent development in proteomics, proteins in the seminal plasma have been more thoroughly investigated. Identification of these proteins has been followed by the investigation to understand their function during fertilization. Especially, seminal plasma proteins that are involved in capacitation have been studied since 1980s (Manjunath and Sairam, 1987; Seidah *et al.*, 1987; Manjunath and Thérien, 2002). Bovine seminal plasma proteins including PDC-109, BSP-A3, and BSP-30 kDa have been reported to stimulate the efflux of cholesterol and phospholi-

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plipds during capacitation (Thérien *et al.*, 1995; Thérien *et al.*, 1999). In addition, proteins from accessory glands affecting the fertility of Holstein bulls were identified. They comprise BSP-30 kDa, osteopontin, phospholipase A2 and Spermadhesin Z13 (Moura, 2005; Souza *et al.*, 2008). The objective of this study was to compare seminal plasma protein profiles between Hanwoo and Korean native brindle cattle bulls and to determine the specific seminal plasma proteins from Hanwoo and Korean native brindle cattle bulls that may affect sperm capacitation and fertilization.

MATERIALS AND METHODS

Experimental Animals and Seminal Plasma Preparation

Four Korean native cattle, Hanwoo, bulls and three Korean native brindle cattle (KNBC) bulls were used to collect semen by artificial vagina. Characteristics of semen samples are shown in Table 1. Semen was divided into 1.5 ml Eppendorf tubes and centrifuged for 10 min at 4℃ with 12,000 rpm to isolate seminal plasma. Seminal plasma was stored at −80℃ until use. Protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as standard. Materials for one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), staining and destaining solutions were purchased from Bio-Rad (Hercules, CA, U.S.A.).

One-Dimensional Gel Electrophoresis

The one-dimension electrophoresis was performed with 14% acrylamide (37.5:1 ratio of acrylamide to bisacrylamide) using Protean Plus Dodeca Cell (BioRad, Hercules, CA, USA) at 200 V and with the starting current (per gel) of 50 mA and final current (per gel) of 33 mA until the line of bromophenol blue reached the bottom of the gel. Twenty μg of semen samples with sample buffer in 20 μl were loaded into each well. Gels were stained by 0.1% colloidal Coomassie Brilliant Blue R-250 (CBBG) for 30min, after destaining with the Destaining solution (Bio-Rad) and were digitalized by an imaging system (VersaDoc MP 5000 system, Bio-Rad).

Isoelectric Focusing (IEF) and 2-Dimensional Electrophoresis (2-DE)

Isoelectric focusing was performed with a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) using 13cm linear range of pH 3~10 immobilized pH gradient (IPG) strip gels (Bio-Rad, USA). Seminal plasma protein (200 μg) samples treated with rehydration buffer (450 μl /strip), containing 8 M urea, 0.2% DTT, and 0.2% Pharmalyte (pH 3~10 linear) were applied to the strip holder. The strip gels were then rehydrated for 12 h. IEF was carried out initially at 250 V for 30 min, increasing the voltage to 10,000 V linearly over 1 h, kept at 10,000 V for 8 h, and then finally at 500 V for 30 min at 50 mA/strip for 70 kVh at 20℃. After IEF separation, the strips were equilibrated in equilibrium buffer A (50 mM Tris-HCl, pH 8.8, 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT) for 15 min, followed by an additional equilibrium for 15min in equilibrium buffer B (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 4.8% iodoacetamide).

The second dimensional gel electrophoresis (2-DE) was performed (Shim *et al.*, 2010) with 15% acrylamide (37.5:1 ratio of acrylamide to bisacrylamide) using Pro-

Table 1. List of experimental animals and characteristics of semen samples used in this study

Identification No.	Breed	Initial of breed	PMOT[1] (%)	MOT[2] (%)	Ejaculate volume (ml)	BSP[3] volume (ml)
2-1031	Hanwoo	HW1	64	74	7	3.8
2010266	Hanwoo	HW2	80	80	7	3.8
2010257	Hanwoo	HW3	90	90	5	2.6
2010264	Hanwoo	HW4	90	90	7	3.8
6-0062	Korean native brindle cattle	KNBC1	77	82	7	3.8
6-8081	Korean native brindle cattle	KNBC2	90	93	5	2.6
C006.4055	Korean native brindle cattle	KNBC3	80	80	7	3.8

[1]PMOT = Proceed motility.
[2]MOT = Motility.
[3]BSP volume = Bovine seminal plasma volume. Amount of seminal plasma collected after centrifugation of semen.

tean Plus Dodeca Cell (BioRad, USA) at 120 V for 30 min and 200 V until the line of bromophenol blue reached the bottom of the gel. Gels were stained by 0.1% colloidal Coomassie Brilliant Blue G-250 (CBBG) in 2% phosphoric acid, 10% ammonium sulphate, and 40% methanol for 48 h (Friedman *et al.*, 2009), and image of the spots for analysis were obtained by Versa-Doc MP 5000 imaging system (Bio-Rad, USA). Two experiments were performed for each Hanwoo seminal plasma samples and three experiments were performed for KNBC seminal plasma samples.

Image Analysis and Spot Identification

The density of all collected protein spots was numerically determined using a PDQuest software program (Bio-Rad, USA) and analyzed. Paired spots for both breeds with varying degrees of expression patterns, and two additional spots for Hanwoo and one spot for KNBC were selected for protein identification. Protein identification was performed by a vender (Protein Works, Daejeon, Korea). Briefly, spots were excised using an automated spot cutter as a circular plug 1.5 mm in diameter. Proteins were subjected to in-gel digestion. MS/MS of peptide generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (AB Sciex instruments, CA, USA). To identify the protein, peptide masses from Q-TOF MS were matched with the theoretical peptides of proteins in the NCBI database using MASCOT search program (www.matrixscience.com).

Statistical Analysis

After 2-DE analysis, density values of the chosen spots for protein identification were compared between Hanwoo and KNBC. The density of the each chosen spot from Hanwoo and KNBC seminal plasma protein were analyzed by GLM procedure of SAS program. To determine the variation in the seminal plasma proteins, the mean density of each chosen spot from bulls were compared using the Duncan's Multiple Range Test with a predetermined significance level of $p<0.05$.

RESULT

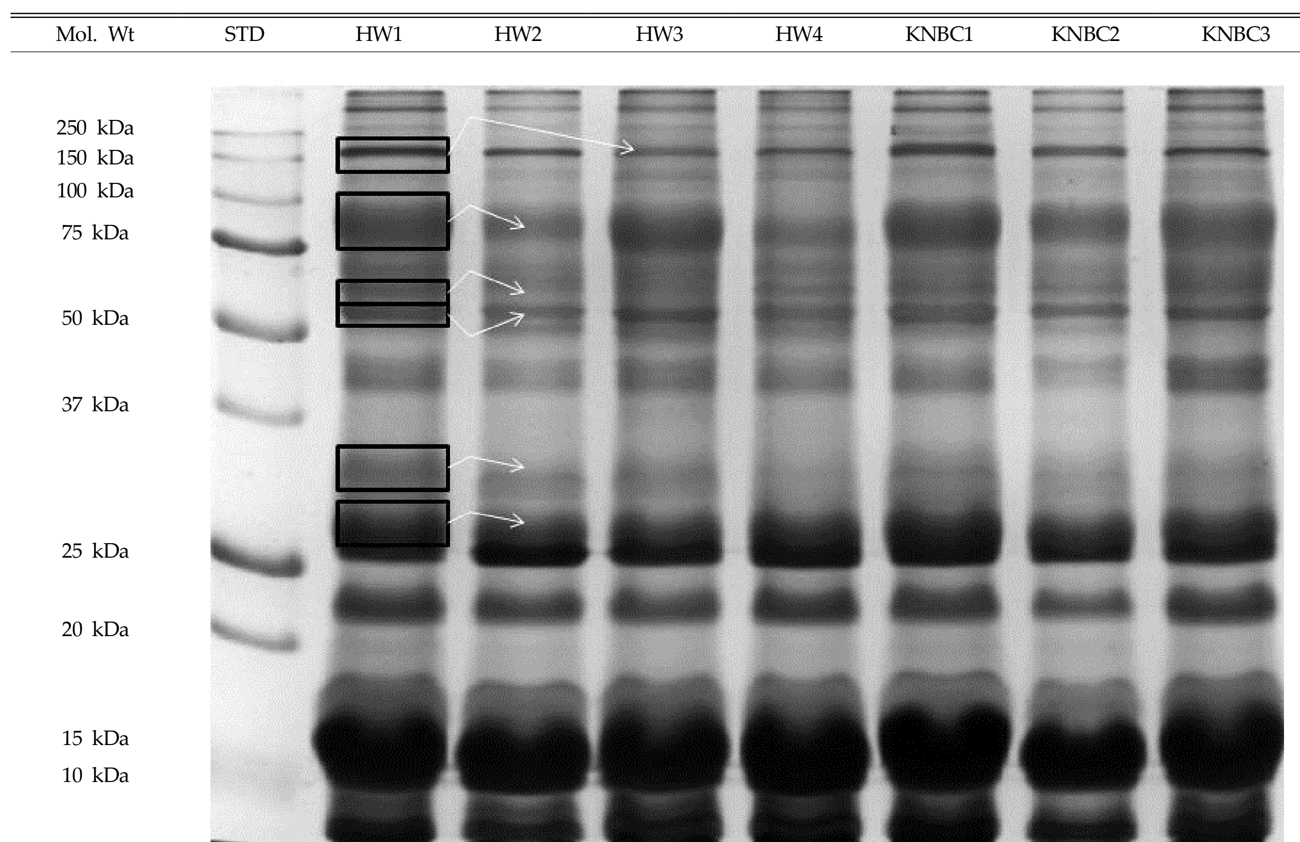


Fig. 1. Representative picture of seminal plasma proteins separated by SDS-PAGE from Hanwoo (HW) and Korean native brindle cattle (KNBC). Picked bands for protein identification were marked by black rectangles. The paired bands showing differential expression are indicated by the arrows for comparison.

One-Dimension Electrophoresis and Protein Identification

Differential expression profile of the seminal plasma was revealed after SDS-PAGE among the animals studied (Fig. 1). There were no clear breed differences in the expression pattern of the seminal plasma between Hanwoo and KNBC, rather there was differential expression of proteins among the bulls included in each breed. Therefore, strong bands with the most variations in Coomassie staining among the bulls, irrespective of their breeds, were chosen to identify the proteins. Molecular weights (MW) of the proteins with clear differences in their expression determined by Coomassie staining were ranged from approximately 25 kDa to 150 kDa. The proteins with MW 50∼55 kDa and 150 kDa emerged as distinct bands, while proteins with MW 25∼30 kDa, 30∼34 kDa, 55∼58 kDa, and 75∼90 kDa appeared as broad collection of proteins (Fig. 1 and 2). The intensity of bands for these proteins were illustrated as density trace (Fig. 2). Proteins from these differentially expressed 6 bands were identified by Q-ToF analysis and are listed in Table 2. Four proteins, one protein from each band, were identified, but no proteins were identified from two other bands. Among the four identified proteins, three were bovine proteins including plasma serine protease inhibitor precursor, platelet-activating factor acetylhydrolase, and complement factor H, and one was an uncharacterized protein.

Two-Dimension Electrophoresis and Protein

Seminal plasma proteins from Hanwoo and Korean native brindle cattle bulls were separated by 2-dimentional SDS-PAGE (Fig. 3). Total number of protein spots from seminal plasma was higher in Korean native brindle cattle 250±24 (mean±SEM) than in Hanwoo 180 ±8. The most highly expressed proteins determined by

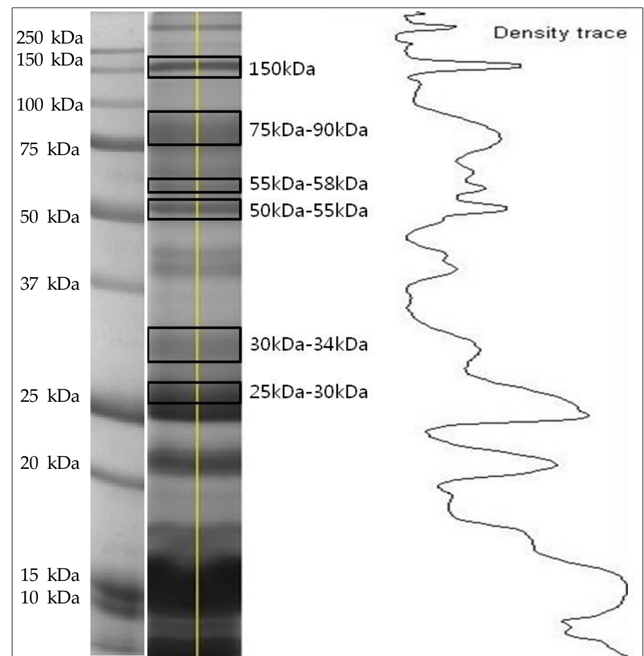
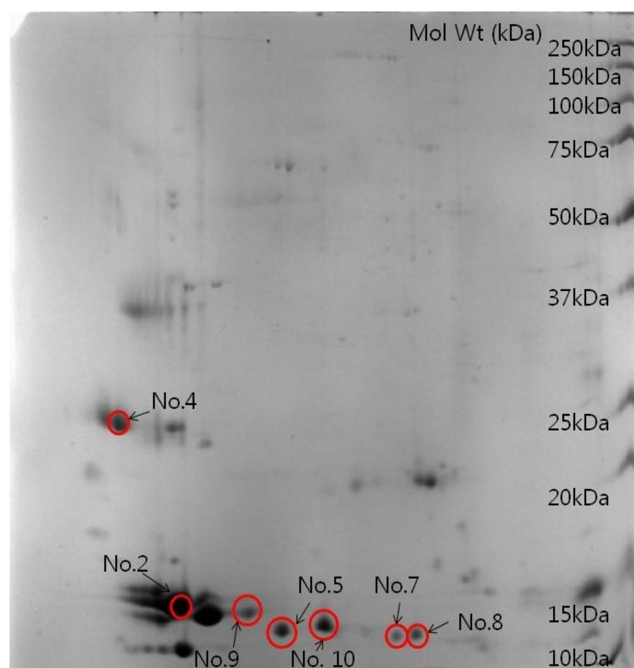


Fig. 2. Tentative molecular weights of the picked bands for protein identification (in black rectangles) from a Hanwoo are shown with the density trace.

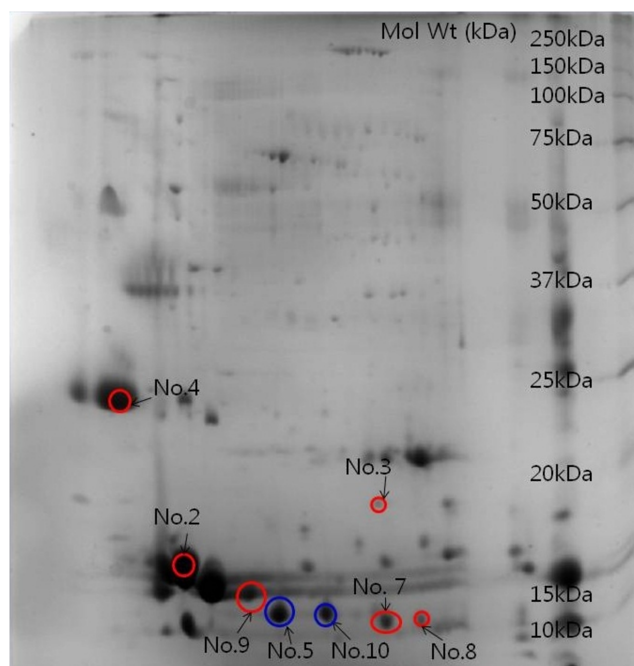
Table 2. List of proteins identified by Q-ToF analysis from the seminal plasma of Hanwoo after SDS-PAGE

Mol. Wt	Protein identified	NCBI gi No.	MW[1]	pI[2]	Source	C/Q[3]	Peptide sequence
150 kDa	Complement factor H	CFAH_BOVIN	144,958	6.43	*Bos taurus*	1/1	453 R.IENGFLSES TFTYPLNK.Q 469
75 kDa∼ 90 kDa	UNIDENTIFIED						
55 kDa∼ 58 kDa	Platelet-activating factor acetylhydrolase	gi|2497684	50,501	6.21	*Bos taurus*	2/1	311 R.IPQPLFFIN SER.F 322
50 kDa∼ 55 kDa	Plasma serine protease inhibitor precursor	gi|28603766	45,439	9.40	*Bos taurus*	2/1	135 R.LPIQEAFL GAMR.T 146
30 kDa∼ 34 kDa	UNIDENTIFIED						
25 kDa∼ 30 kDa	Uncharacterized protein	G3VW07	16,087		*Sarcophilus laniarius*		[4]**PVNTF**V**HE**TV**W**VK

[1] Mol. Wt = molecular weight.
[2] pI = isoelectric point.
[3] C/Q = sequence coverage/queries matched.
[4] Matched amino acids are shown in **bold** and underlined.



(A) Hanwoo



(B) Korean native brindle cattle

Fig. 3. Representative picture 1of the 2-demensional gel electrophoresis of seminal plasma proteins from four Hanwoo (A) and three Korean native brindle cattle (B). Spots picked for protein identification include the paired spots (spots 2, 4, 7, 8, and 9) for both breeds, additional spots for Hanwoo (spots 5 and 10), and one spot for Korean native brindle cattle (spot 3). Picked spots for protein identification are circled in red and marked by the arrows. Tentative paired spots (5 and 10) for Korean native brindle cattle are circled in blue and marked by the arrows.

Coomassie Brilliant Blue staining were proteins with MW between 10∼15 kDa and 20∼25 kDa. Differential expression pattern of seminal plasma proteins shown by 1-dimentional SDS-PAGE were not clearly discernible after 2-DE. Therefore, paired spots (spots 2, 4, 7, 8 and 9) for both breeds with varying degrees of expression patterns, and additional spots 5 and 10 from Hanwoo, and spot 3 from KNBC were selected for protein identification and density comparison (Fig. 3). The list of proteins identified is shown in Table 3. Three proteins (spots 2, 4 and 9) were identified out of five paired spots with amino acid sequence match for both Hanwoo and Korean native brindle cattle. Interestingly, spots 2 and 9 were identical protein of chain A, bull seminal plasma PDC-109 Fibronectin Type II module, though different parts of the protein were matched. The paired spot 4 was identified as the seminal plasma protein BSP-30 kDa precursor with matching amino acid sequences between Hanwoo and KNBC. The paired spot 7 was identified as the Spermadhesin Z13 in KNBC or its precursor in Hanwoo with different amino acid sequences. The paired spot 8 appeared to be in the same location for both breeds. However, the spot 8 was identified as the Spermadhesin Z13 precursor in Hanwoo or Spermadhesin-1 in KNBC. The amino acid sequence of Spermadhesin Z13 precursor in Hanwoo from spot 8 was identical to that of the Spermadhesin Z13 precursor from spots 7. Out of the 2 additional unpaired spots from Hanwoo, both spots 5 and 10 were identified as the Spermadhesin Z13 precursor and its amino acid sequence was identical to that of the Spermadhesin Z13 precursor from spot 7 in Hanwoo. Therefore, spots 5, 7, 8 and 10 were the Spermadhesin Z13 precursor in Hanwoo. The remaining spot 3 from KNBC was prominent just below 20 kDa and it was identified as the seminal plasma protein A3. The spot 3 was not visible in Hanwoo and therefore, was not identified.

The density of the each paired spot that was identified as the same protein for both breed were compared between Hanwoo and KNBC. When the densities of the chain A, bull seminal plasma PDC-109 Fibronectin Type II module for spots 2 and 9 (Fig. 4A and 4B) were compared between the two breeds, the protein from KNBC had significantly higher density than Hanwoo (p<0.05). Both breeds have large quantities of the protein in the seminal plasma. The density of the seminal plasma protein BSP-30 kDa (spot 4, Fig. 4C) was significantly higher (p<0.05) in KNBC than Hanwoo. Hanwoo 3 had comparable level of BSP-30 kDa to KNBC. The density of the Spermadhesin Z13 and precursor (spot 7, Fig. 4D) was significantly higher (p< 0.05) in KNBC than Hanwoo. The variation of the density within the KNBC appears to be minimal, but Hanwoo 2 had reduced density. The variation of the density within the breeds appears to be minimal for cha-

Table 3. List of protein identified by Q-ToF analysis from Hanwoo and Korean native brindle cattle after 2-dimensional gel electrophoresis

Spot No.	Protein identified	NCBI gi No.	MW[1)]	pI[2)]	Source	C/Q[3)]	Peptide sequence
HW No. 2	Chain A, Bull Seminal Plasma PDC-109 Fibronectin Type II Module	gi|20663779	13,244	5.08	*Bos taurus*	26/12	86 K.IGSMWMSWC SLSPNYDKDR.A 104
KNBC No. 2	Chain A, Bull Seminal Plasma PDC-109 Fibronectin Type II Module	gi|20663779	13,244	5.08	*Bos taurus*	17/1	86 K.IGSMWMSWC SLSPNYDKDR.A 104
KNBC No. 3	Seminal plasma protein A3	gi|297469372	16,657	7.42	*Bos taurus*	11/1	72 R.DSIFLWCSL SADYTGR.W 87
HW No. 4	Seminal plasma protein BSP-30 kDa precursor	gi|28849953	21,711	5.73	*Bos taurus*	10/1	85 K.ANDLNAVFEG PACAFPFTYK.G 104
KNBC No. 4	Seminal plasma protein BSP-30 kDa precursor	gi|28849953	21,711	5.73	*Bos taurus*	10/1	85 K.ANDLNAVFEG PACAFPFTYK.G 104
HW No. 5	Spermadhesin Z13 precursor	gi|126158907	15,496	5.92	*Bos taurus*	17/1	71 R.DVHLNCNKESL EIIEGPPESSNSR.K 94
HW No. 7	Spermadhesin Z13 precursor	gi|126158907	15,496	5.92	*Bos taurus*	17/1	71 R.DVHLNCNKESL EIIEGPPESSNSR.K 94
KNBC No. 7	Spermadhesin Z13	gi|12585540	13,660	5.58	*Bos taurus*	16/1	78 K.ICDTSHAEYT SCTNTMTVK.Y 96
HW No. 8	Spermadhesin Z13 precursor	gi|126158907	15,496	5.92	*Bos taurus*	17/1	71 R.DVHLNCNKESL EIIEGPPESSNSR.K 94
KNBC No. 8	Spermadhesin-1	gi|297464530	15,265	8.37	*Bos taurus*	14/1	116 R.SSNQPVSSFD IFYYGRPSA.-134
HW No. 9	Chain A, Bull Seminal Plasma PDC-109 Fibronectin Type II Module	gi|20663779	13,244	5.08	*Bos taurus*	28/1	1 -.DQDEGVSTEP TQDGPAELPEDE ECVFPFVYR.N31
KNBC No. 9	Chain A, Bull Seminal Plasma PDC-109 Fibronectin Type II Module	gi|20663779	13,244	5.08	*Bos taurus*	37/9	1 -.DQDEGVSTEP TQDGPAELPED EECVFPFVYR.N 31
HW No. 10	Spermadhesin Z13 precursor	gi|126158907	15,496	5.92	*Bos taurus*	17/1	71 R.DVHLNCNKESL EIIEGPPESSNSR.K 94

[1)] MW = molecular weight.
[2)] pI = isoelectric point.
[3)] C/Q = sequence coverage/queries matched.

in A, bull seminal plasma PDC-109 Fibronectin Type II module, BSP-30 kDa, and Spermadhesin Z13 or its precursor.

When we compared the additional spot 5 between the two breeds (though we did not identify it in KN-BC), there were two bulls with low density and two bulls with high density in Hanwoo (Fig. 4F). KNBC bulls had higher density than Hanwoo bulls. When we compared the additional spot 10 between the two breeds (though we did not identify it KNBC), two KNBC bulls had higher density than Hanwoo bulls, but one KNBC bull had similar density to Hanwoo bulls (Fig. 4H).

DISCUSSION

The objective of this study was to compare seminal plasma protein profiles between Hanwoo and Korean native brindle cattle bulls and to determine the specific seminal plasma proteins from Hanwoo and Korean native brindle cattle bulls that may affect sperm capacitation and subsequent fertilization. Seminal plasma was isolated from the semen of Korean native cattle, Hanwoo, and Korean native brindle cattle bulls. Total number of spots from seminal plasma of the Korean native brindle cattle was about 250, which was similar to the

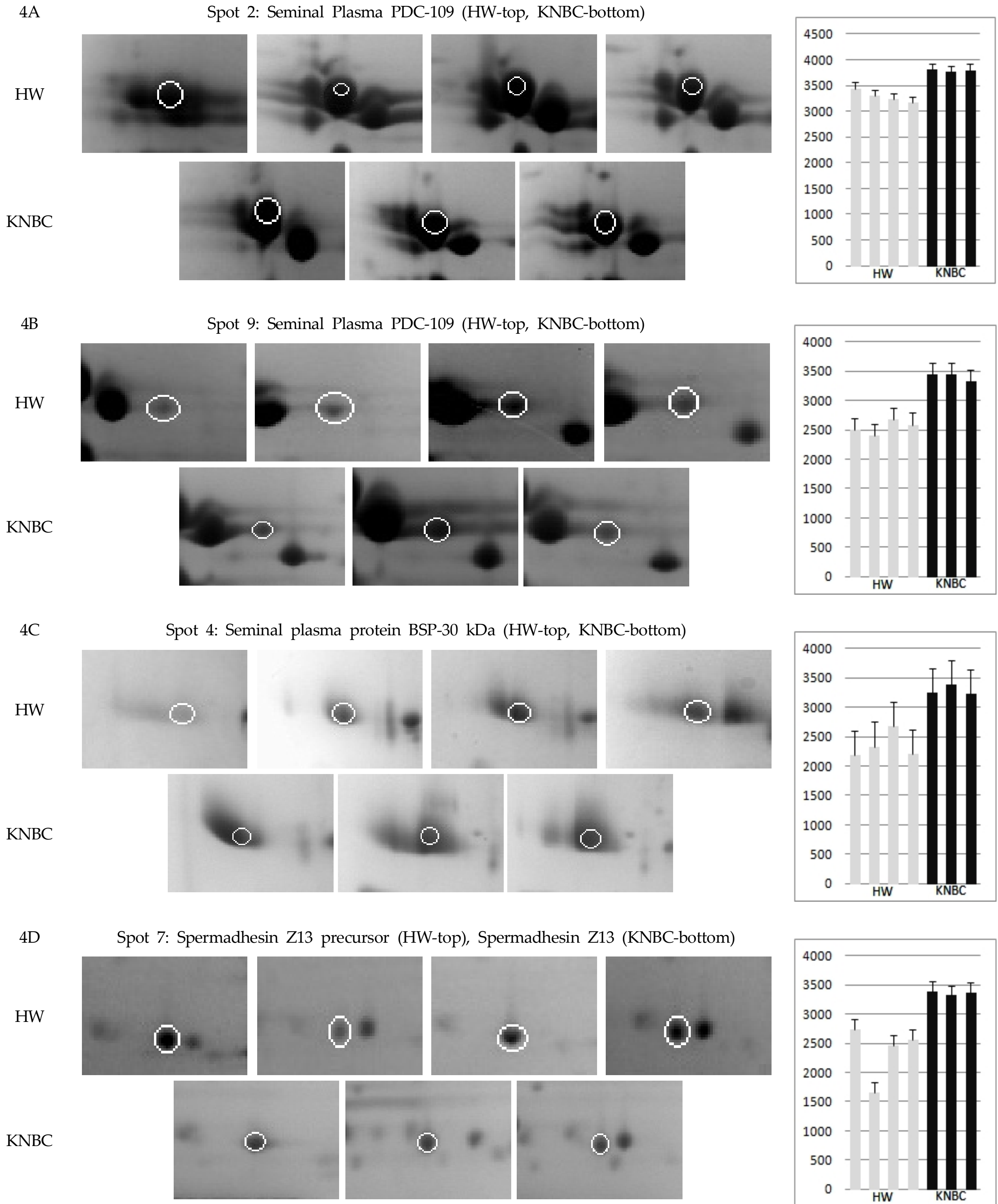


Fig. 4. In the left panel, expression profiles of each protein identified from four Hanwoo (HW-top) and three Korean native brindle cattle (KNBC-bottom) bulls between MW 10 kDa～30 kDa in the seminal plasma are shown. Circles in white show the spots used for density calculation by PDQuest software. In the right panel, the average and standard error mean of density of spots for four Hanwoo (HW-gray bar) and three Korean native brindle cattle (KNBC-black bar) are shown. Not visible for spot 3 in HW due to low density (4E).

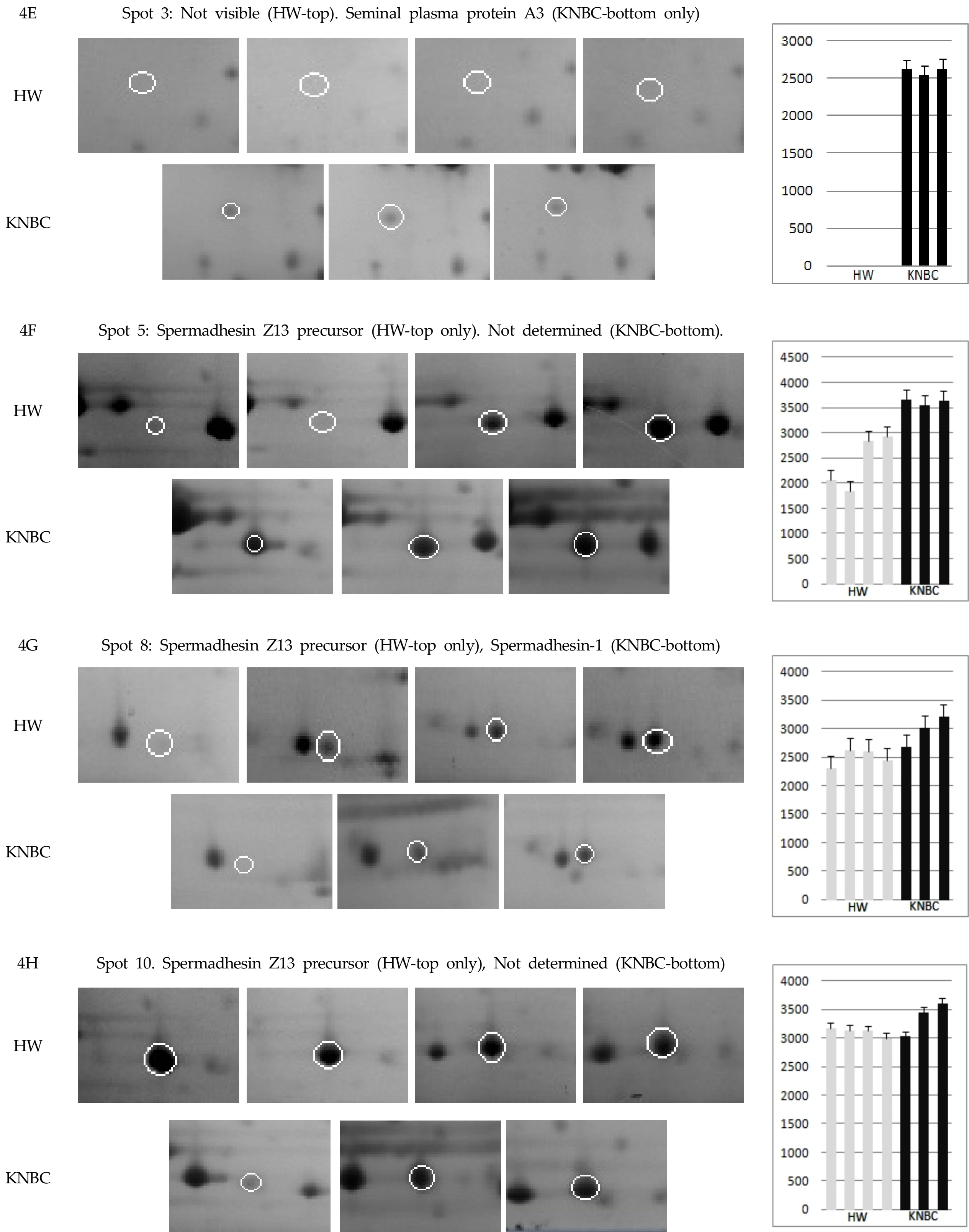


Fig. 4. Continued.

report of Kelly *et al.*,(2006) that showed approximately 250 protein spots. But they were less in Hanwoo with approximately 180 spots. This may need to be confirmed using additional bulls.

Out of the 3 identified proteins and 1 uncharacterized protein after one-dimensional SDS-PAGE, the largest protein identified was complement factor H. Complement factor H was from the 150 kDa band that was distinct and the MW of the band was comparable to the known MW of 144,958. Complement factor H is known to control the function of the alternative complement pathway. However, the origin and the importance of this protein in the seminal plasma have not been described. The second largest protein that was identified was PAF-activating factor acetylhydrolase (PAF-AH). It was from the 55～58 kDa band that was broad and the MW of the band was close to the known MW of 50,501. PAF-AH is the naturally occurring enzyme that converts PAF to biologically inactive Lyso-PAF (Bathgate *et al.*, 2007). When swine sperm were frozen in the presence of PAF-AH, acrosome integrity was higher immediately after thawing and after 3 and 6 h incubation at 37℃. It is also present in the bovine seminal vesicle fluid (Hough and Parks, 1997). The third protein identified was the plasma serine protease inhibitor precursor. It was from the 50～55 kDa band, which was distinct and the MW of the band was close to the known MW of 45,439. One of the plasma serine protease inhibitor known as SERPINE2 could inhibit *in vitro* bovine serum albumin-induced sperm capacitation, prevent sperm binding to the egg, and thus blocking fertilization in mice (Lu *et al.*, 2011). The function of this protein in cattle has not been studied regarding fertilization. The previously reported aspects of PAF-AH and plasma serine protease inhibitor can be both beneficial and detrimental for the fertilization process. Therefore, applying different functional aspects of the identified seminal plasma proteins for storage, capacitation and fertilization may affect fertility in cattle. One uncharacterized protein with homology to G3VW07 (MW 16,087) of Sarcophilus laniarius may need to be studied in the future.

For further analysis of bovine seminal plasma proteins, they were separated by 2-DE. Interestingly, variation noted between Hanwoo and KNBC, especially from 25～30 kDa to 150 kDa, after one-dimensional SDS-PAGE was not very clearly distinguished. This may be because the bands separated by one-dimensional SDS-PAGE may have more than one protein, though only one protein per band was identified or the amount of the protein may have been too low for those spots to be visualized by 2-DE. There were strong Coomassie staining of proteins that were clustered near 10～15 kDa, which were not properly separated by one-dimensional SDS-PAGE (14%). After separation by 2-DE, proteins that were clustered near 10～15 kDa after one-dimensional SDS-PAGE were clearly visible and some of them were selected for identification. Spots 2 and 9 were identified as the identical protein of the chain A, bull seminal plasma PDC-109 Fibronectin Type II module for both Hanwoo and KNBC with known MW of 13,244 Da. Though spot 2 appeared to have higher MW than spot 2 and spot 2 was larger than spot 9, both spots were identified as the same protein. PDC-109 is one of the three major heparin-binding proteins in the bull seminal plasma and it is also known as BSP-A1/A2. It was noted that PDC-109 binds to sperm surface choline lipids and promotes sperm capacitation by stimulating the efflux of cholesterol and phospholipids in cattle (Wah *et al.*, 2002). PDC-109 plays a role as a sperm reservoir where sperm bind to oviductal epithelium (Gwathmey *et al.*, 2003; Gwathmey *et al.*, 2006) and as a molecular chaperone (Sankhala and Swamy, 2010). The parts of amino acid sequences that matched PDC-109 Fibronectin Type II are different between spot 2 and 9. It is not known whether PDC-109 is produced with different MW. Presence of these two spots for the same protein may be due to post-translational modification, since it was reported as glycoprotein (Manjunath and Thérien, 2002). The functional differences of these two forms of PDC-109 may require further studies.

The spot 4 was identified as the seminal plasma protein BSP-30 kDa precursor for both Hanwoo and KNBC. BSP-30 kDa was previously reported as one of the major proteins in the bovine seminal plasma (Manjunath and Thérien, 2002). Spot 4 was the most prominent protein in both Hanwoo and KNBC between MW of 20～30 kDa. BSP-30 kDa share its functional role of PDC-109 in storing sperm in the oviduct (Gwathmey *et al.*, 2006) and it was previously considered as being associated with high fertility.

The spot 7 was identified as the Spermadhesin Z13 in KNBC or its precursor in Hanwoo of the protein. When we analyzed spots 5 and 10 in Hanwoo (proteins were not determined for paired spots 5 and 10 for KNBC), spots 5 and 10 were identified as the Spermadhesin Z13 precursor. The spot 8 was also identified as the Spermadhesin Z13 precursor in Hanwoo. Therefore, there were 4 different spots (5, 7, 8 and 10) for the same protein, Spermadhesin Z13 precursor, in Hanwoo. We identified Spermadhesin Z13 for spot 7 in KNBC and we suspect that spots 5 and 10 may be the Spermadhesin Z13, though we did not determine spots 5 and 10. Interestingly, the spot 10 was bigger than spot 5 in Hanwoo, while the tentative spot 5 appeared bigger than spot 10 in KNBC. It is likely they have different post-translational modification including glyco-

sylation as their MW appeared to be similar. Odhiambo and Dailey (2011) reported that two spots for Spermadhesin Z13 in Holstein bulls. Therefore, Spermadhesin Z13 may exist in multiple/different forms in different breeds. It appeared that there was a variation in density of the spot 5 among Hanwoo. Two bulls with lower motility had lower density for Spermadhesin Z13 precursor in spot 5. But, there was little variation in the density of spot 10. Killian *et al.* (1993) suggested that Spermadhesin Z13 was associated with the low fertility. However, Odhiambo and Dailey (2011) did not find that Spermadhesin Z13 was associated with the low fertility and it was classified as one of the membrane stabilizing proteins. It was reported that Spermadhesin Z13 is present as nonglycosylated dimer in solution (Tedeschi *et al.*, 2000). Whether variation of Spermadhesin Z13 precursor had implication in fertility in cattle require further studies.

The spot 8 was identified as the Spermadhesin-1 in KNBC. Spermadhesin-1 is a seminal plasma protein that was mapped to bovine chromosome 26q23 (Masabanda *et al.*, 1999). The function of this protein is not well known.

The spot 3 from KNBC was prominent just below 20 kDa and it was identified as the seminal plasma protein A3 (BSP-A3) with known MW of 16,657. It is one of the major seminal proteins that was identified with BSP-A1, BSP-A2, and BSP-30 kDa (Manjunath and Thérien, 2002). BSP-PDC109, BSP-A3 and BSP-30 kDa, which together constitute about half of the total protein, was reported as the three major proteins of bovine seminal plasma (Kelly *et al.*, 2006). It was surprising to find that the spot was not visible in Hanwoo. We may need to look at seminal plasma of other Hanwoo bulls whether this is authentic observation. If so, the lack of BSP-A3 in Hanwoo may warrant further investigation.

We have investigated the expression of seminal plasma protein from Hanwoo and KNBC. The differential expression of some of the proteins was intriguing. Furthermore, presence of the multiple forms of plasma proteins, especially Spermadhesin Z13 suggests that they may have specific effects on sperm function, capacitation and fertilization. After the crystal structure of plasma proteins become available, including PDC-109, the function and binding characteristics of ligands can be explored. Several of the plasma proteins that we identified from Hanwoo and KNBC are involved in sperm capacitation, especially through the mechanism of cholesterol efflux. Sperm function may be improved and fertility may be increased with the optimal regulation of cholesterol efflux by the seminal plasma proteins and their ligands that may increase or decrease cholesterol transport from sperm. Studies on the mechanism of action, utilization of ligands and the variation of the plasma proteins may help improving fertility in cattle.

REFERENCES

1. Bathgate R, Maxwell WM, Evans G (2007): Effects of platelet-activating factor and platelet-activating factor: acetylhydrolase on *in vitro* post-thaw boar sperm parameters. Theriogenology 67:886-892.
2. Friedman DB, Hoving S, Westermeier R (2009): Isoelectric focusing and two-dimensional gel electrophoresis. Methods Enzymol. 463:515-540.
3. Gwathmey TM, Ignotz GG, Suarez SS (2003): PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium *in vitro* and may be involved in forming the oviductal sperm reservoir. Biol Reprod 69:809-815.
4. Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS (2006): Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. Biol Reprod 75:501-507.
5. Hough SR, Parks JE (1997): Partial purification and localization of platelet-activating factor acetylhydrolase from bovine seminal plasma. J Androl 18: 540-548.
6. Kelly VC, Kuy S, Palmer DJ, Xu Z, Davis SR, Cooper GJ (2006): Characterization of bovine seminal plasma by proteomics. Proteomics 6:5826-5833.
7. Killian GJ, Chapman DA, Rogowski LA (1993): Fertility-associated proteins in Holstein bull seminal plasma. Biol Reprod 49: 1202-1207.
8. Lu CH, Robert L, Lee KK, Hwu YM, Chu SL, Chen YJ, Chang WC, Lin SP, Li SH (2011): SERPINE2, a serine protease inhibitor extensively expressed in adult male mouse reproductive tissues, may serve as a murine sperm decapacitation factor. Biol Reprod 84:514-525.
9. Manjunath P, Sairam MR (1987): Purification and biochemical characterization of three major acidic proteins (BSP-A1, BSP-A2 and BSP-A3) from bovine seminal plasma. Biochem J 241:685-692.
10. Manjunath P, Thérien I (2002): Role of seminal plasma phospholipid - binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 53:109-119.
11. Masabanda J, Buitkamp J, Ewald D, Einspanier R, Fries R (1999): Molecular markers for the bovine gene encoding acidic seminal fluid protein precursor (spermadhesin 1, SPADH1) map to chromosome 26q23. Anim Genet 30:396-407.
12. Moura AA (2005): Seminal plasma proteins and fertility indexes in the bull: The case for osteopontin. Anim Reprod J 2:3-10.
13. Odhiambo J, Dailey R (2011): Characterization of proteins in cryopreserved and non-cryopreserved seminal plasma of dairy bulls of differing fertility. OJAS 1:33-40.

14. Sankhala RS, Swamy MJ. (2010): The major protein of bovine seminal plasma, PDC-109, is a molecular chaperone. Biochem 49:3908-3918.
15. Seidah NG, Manjunath P, Rochemont J, Sairam MR, Chrétien M (1987): Complete amino acid sequence of BSP-A3 from bovine seminal plasma. Homology to PDC-109 and to the collagen-binding domain of fibronectin. Biochem J 243:195-203.
16. Shim KS, Park GH, Hwang IH (2010): Proteomics comparison of longissimus muscle between Hanwoo and Holstein cattle. KOSFA 30:385-391.
17. Souza CE, Moura AA, Monaco E, Killian GJ (2008): Binding patterns of bovine seminal plasma proteins A1/A2, 30 kDa and osteopontin on ejaculated sperm before and after incubation with isthmic and ampullary oviductal fluid. Anim Reprod Sci 105: 72-89.
18. Tedeschi G, Oungre E, Mortarino M, Negri A, Maffeo G, Ronchi S (2000): Purification and primary structure of a new bovine spermadhesin. Eur J Biochem 267:6175-6179.
19. Thérien I, Bleau G, Manjunath P (1995): Phosphatidylcholine binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. Biol Reprod 52:1372-1379.
20. Thérien I, Moreau R, Manjunath P (1999): Bovine seminal plasma phospholipid-binding proteins stimulate phospholipid efflux from epididymal sperm. Biol Reprod 61:590-598.
21. Wah DA, Fernández-Tornero C, Sanz L, Romero A, Calvete JJ (2002): Sperm coating mechanism from the 1.8 A crystal structure of PDC-109-phosphorylcholine complex. Structure 10:505-514.

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