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Original Article

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Abstract

Our study investigated proteins in bovine spermatozoa using 2-DE and LC MS/MS and found a markedly different expression in mature bovine proacrosin binding protein (sp32), which is the focus of the present study. Spermatozoa extract from nine fertile Brahman bulls was separated using 2-DE followed by Coomassie brilliant blue staining. From over 600 protein spots, a particular protein spot with two different patterns was detected at 32 kDa. The high relative protein content of spot Y_1 (32 kDa pI 5.3, pattern A) was found in six bulls whereas the remaining three bulls expressed two small spots Y_1 and Y_2 (32 kDa pI 5.3 and 31.5 kDa pI 5.5, respectively; pattern B). Identification of spots Y_1 and Y_2 both in pattern A and B, by using LC MS/MS, revealed the predicted protein (derived from a genomic sequence)—the proacrosin binding protein, sp32 (62.3 kDa and pI 5.1) found in *Bos taurus*. The amino acid sequence coverage of these spots corresponded to the carboxyl-terminal half of sp32. Immunoblotting and immunocytochemistry with the anti-phosphotyrosine antibody were performed to determine whether the spot Y_2 was a tyrosine phosphorylated form (p32) of sp32 as reported on *Sus scrofa*. The results obtained did not confirm that Y_2 was a p32 form of sp32. In conclusion, we applied proteomic approaches to characterize bovine spermatozoa proteins and reported a mature bovine sp32, with a relative molecular mass of ~32 kDa. The markedly different expressions of sp32 were investigated for any post-translational modification, specifically phosphorylation; however, phosphotyrosine protein (p32) could not be confirmed. Further characterization and functional analysis are needed.

Keywords: bovine spermatozoa proteins, 2-DE, LC MS/MS, sp32

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บทคัดย่อ

การแสดงออกของ proacrosin binding protein (sp32) ของโค ในรูปแบบที่แตกต่างกัน

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งานวิจัยนี้ทำการศึกษาโปรตีนในเซลล์อสุจิของโคโดยใช้เทคนิค 2-DE และ LC MS/MS และค้นพบประเด็นที่น่าสนใจ คือ การแสดงออกของ proacrosin binding protein (sp32) ที่มีรูปแบบแตกต่างกัน จากการแยกโปรตีนของเซลล์อสุจิของพ่อโคพันธุ์บรามันห์ที่มีความสมบูรณ์พันธุ์ จำนวน 9 ตัว ด้วยเทคนิค 2-DE และย้อมด้วยสี Coomassie brilliant blue พบจุดโปรตีนมากกว่า 600 จุด บนแผ่นเจล จากจุดโปรตีนทั้งหมดนี้ตรวจพบความแตกต่างของโปรตีนที่มีขนาด 32 กิโลดาลตัน ใน 2 รูปแบบ คือ รูปแบบ A และ B โดยรูปแบบ A มีการแสดงออกของโปรตีนที่จุด Y_1 (ขนาด 32 กิโลดาลตัน และมีค่า pI เท่ากับ 5.3) ในปริมาณมาก ซึ่งตรวจพบในพ่อโคจำนวน 6 ตัว ส่วนรูปแบบ B มีการแสดงออกของโปรตีนที่จุด Y_1 (ขนาด 32 กิโลดาลตัน และมีค่า pI เท่ากับ 5.3) และ Y_2 (ขนาด 31.5 กิโลดาลตัน และมีค่า pI เท่ากับ 5.5) ในปริมาณน้อย ซึ่งตรวจพบในพ่อโคจำนวน 3 ตัว หลังจากทำการจำแนกชนิดโปรตีนด้วยเทคนิค LC MS/MS พบว่า โปรตีนจุด Y_1 และ Y_2 ทั้งในรูปแบบ A และ B คือ predicted (ได้มาจากการคำนวณจากลำดับเบสของจีน): proacrosin binding protein (sp32) ที่มีขนาด 62.3 กิโลดาลตันและมีค่า pI เท่ากับ 5.1 ใน *Bos taurus* โดยที่ลำดับกรดอะมิโนทั้งหมดของโปรตีนจุด Y_1 และ Y_2 ที่ได้จากการทำ LC MS/MS ตรงกันกับลำดับกรดอะมิโนในส่วนครึ่งปลายด้าน carboxyl-terminal ของโปรตีน sp32 จากผลการทดลองที่พบการแสดงออกของโปรตีน sp32 ใน 2 รูปแบบ ทำให้มีศึกษาต่อเนื่องเพื่อตรวจสอบว่า โปรตีน sp32 ที่ปรากฏที่จุด Y_2 เป็นโปรตีนที่อยู่ในรูปแบบของ tyrosine phosphorylation (p32) เหมือนที่เคยมีรายงานไว้ในสุกรหรือไม่ ผลการทำอิมมูนโนบลอต และอิมมูนโนไซโตเคมีด้วยแอนติบอดี anti-phosphotyrosine ไม่สามารถบอกได้ว่าจุดโปรตีน Y_2 คือ p32 จากการศึกษาครั้งนี้จึงสรุปได้ว่า การแยกโปรตีนในเซลล์อสุจิของโคด้วยเทคนิคทางโปรติโอมิกส์ สามารถระบุได้ว่าโปรตีน sp32 มีขนาด 32 กิโลดาลตัน และมีการแสดงออกที่แตกต่างกัน 2 รูปแบบ แม้ว่าการศึกษาครั้งนี้ไม่สามารถชี้ให้เห็นถึงโปรตีน sp32 ในรูปแบบของ p32 อย่างไรก็ตาม เป็นเรื่องน่าสนใจที่จะทำการศึกษาต่อไปว่า การแสดงออกของโปรตีนที่แตกต่างกันนี้จะมีผลต่อการทำหน้าที่ของเซลล์อสุจิ และมีผลต่อการสืบพันธุ์ของพ่อโคอย่างไร

คำสำคัญ: โปรตีน เซลล์อสุจิโค, 2-DE, LC MS/MS, sp32

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Introduction

Spermatozoa possess a specific set of proteins that are needed to fertilize the female gamete. These proteins, located in various parts of spermatozoa, have specific functions; those at the head are involved in binding to oocyte (Moos et al., 1993; Baba et al., 1994; Howes et al., 2001; Kim et al., 2001[a,b]) whereas those in the principle piece of flagella are responsible for flexibility and motility (Krisfalusi et al., 2006; Amos, 2008; Dashina et al., 2010). The characterization of spermatozoa proteins by using proteomic technologies such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) can provide an understanding of spermatozoa function (Pixton et al., 2004; Martínez-Heredia et al., 2006; de Mateo et al., 2007). For example, Martínez-Heredia et al. (2006) reported that the function of 98 different proteins isolated from the 2-DE gels and identified by MS in human normozoospermic spermatozoa was involved in energy production, metabolism, cytoskeleton, cell movement, etc.

The study of spermatozoa proteins has been done mostly in mice and humans for understanding of the defective cause of male infertility (Pixton et al., 2004; Hernandez-Gonzalez et al., 2005; Hu et al., 2006; Roy et al., 2007; Toure et al., 2007). A few studies have demonstrated proteomics analysis of bull spermatozoa using 2-DE and MS, but were primarily comparisons of protein expression profiles among high and low fertility bulls (Peddinti et al., 2008; D'Amours et al., 2010). The objective of the study was to evaluate the bovine spermatozoa proteins by using 2-DE and MS techniques to understand the biochemical aspects of the proteins. This investigation may provide applications to improve bovine reproduction.

Materials and Methods

Experimental animals: Nine Brahman bulls, at the Northeastern Bull Center, Thailand, were used in this study. The bulls were between 4 and 8 years of age. All bulls were proven to have normal fertility, by breeding soundness examination (BSE), as per minimum requirements of the Society for Theriogenology (Chenoweth et al., 1993). The animals were fed concentrated feed and roughage. Only healthy bulls on the day of semen collection were included in the experiment.

Preparation of spermatozoa extract: Semen samples were collected by using an artificial vagina. The first ejaculated semen was used in this study. After collection, 1.0 ml of semen from each bull was centrifuged at 800 *g* for 5 min and then washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) 2 times. The pellet was then lysated with 500 µl of lysis buffer (7M urea, 2M thiourea, 4% w/v CHAPS, 2% w/v DTT) containing protease inhibitor. The mixture was sonicated for 15 sec 3 times at 50% duty cycle at an output control of 5 using Branson 450 Sonifier from G. Heinemann (Schwäbisch Gmünd, Germany), then centrifuged at 10,000 *g* for 60 min at 4^0C. The supernatant was transferred into 1.5 ml tubes and stored at -70^0C for future analysis of protein content and 2-DE.

Spermatozoa protein separation by 2-DE: Before performing 2-DE, the spermatozoa extract was assayed for protein concentration (Bradford, 1976) using BSA as the standard. The 2-DE was performed according to O'Farrell's method (O'Farrell, 1975). Briefly, a total spermatozoa extract protein of 340 µg was diluted in 450 µl of re-hydration solution [7M urea, 2M thiourea, 4% w/v CHAPS, 2% w/v DTT, 0.5% v/v IPG buffer (pH 3-10), 0.002% Bromophenol blue]. This mixing solution was loaded to the Immobiline DryStrip Re-swelling tray from Amersham Biosciences (Uppsala, Sweden); the 24-cm IPG dry strip with a pH range 3-10 was placed with the gel side down and overlaid with an IPG cover fluid. The IPG dry strip was allowed to re-hydrate overnight (10-20 hours) and then transferred to Manifold tray from Amersham Biosciences (Uppsala, Sweden).

Proteins were focused in an Ettan IPGphor II Isoelectric Focusing System from Amersham Biosciences (Uppsala, Sweden) for 7 hours and 30 min with 50 µA per strip in a voltage gradient: (i) step and hold 500 V for 500 Vh (ii) gradient up to 1,000 V for 800 Vh (iii) gradient up to 8,000 V for 13,500 Vh and (iv) step and hold 8,000 V for 20,000 Vh. The focused strip was equilibrated in a buffer (6M urea, 2% w/v SDS, 75 mM Tris-HCl, pH 8.8, 29.3% v/v glycerol, and 0.002% Bromophenol blue) containing 1% w/v DTT for 30 min then changed to equilibrate in a buffer containing 2.5% w/v IAA for 30 min.

After equilibration, the strip was done in a second dimension of 13.5% SDS-polyacrylamide gel as described by Laemmli (1970) using Ettan DALTsix from GE Healthcare (Uppsala, Sweden). The low-molecular weight standard, range 14.4–97 kDa, was loaded to the gel. The vertical setup was used for 4 gels per plate, using 2 W per gel overnight. Electrophoretic separation was done in Tris-glycine buffer, pH 8.3 (25 mM Tris, 192 mM glycine, 0.1% w/v SDS). The gel was fixed (in 5% phosphoric acid, 50% ethanol) for 1 hour and stained in colloidal Coomassie Brilliant Blue (CBB: 0.08% CBB, 8% ammonium sulfate, 0.8% phosphoric acid, 20% methanol) overnight. The gel was de-stained with de-ionized water for at least 24 hours or until the background cleared. At least one duplicate gel was performed for each sample. The CBB-stained gels were scanned with an ImageScanner System and analyzed spots by ImageMaster™ 2D Platinum software from GE Healthcare (Uppsala, Sweden).

A 7-cm long, IPG dry strip (pH range, 3–10) was used to separate the spermatozoa extract for immunoblotting. The 60 µg of spermatozoa extract protein was diluted in 125 µl of re-hydration solution. This mixing solution was loaded to the strip holder from Amersham Biosciences (Uppsala, Sweden); the IPG dry strip was placed with the gel side down and overlaid with an IPG cover fluid. The IPG dry strip was allowed to re-hydrate overnight (10-20 hours). The focus was adjusted according to the IPG strip length steps: (i) step and hold 300 V for 200 Vh (ii) gradient up to 1,000 V for 300 Vh (iii) gradient up to 5,000 V for 4,000 Vh and (iv) step and hold 5,000 V for 2,000 Vh. SDS-PAGE was done on 13.5% polyacrylamide gel as described by Laemmli (1970) and run in the miniVE vertical electrophoresis system from Amersham Biosciences (Uppsala, Sweden). The electrophoresis was run for 1 hour and 30 min at a current constant of 20 mA per gel.

LC MS/MS analysis: The interested protein spots were cut from the gel and sent to the GENOME Institute, Thailand, for identification using the liquid chromatography tandem mass spectrometry (LC MS/MS) analysis. The spot samples were digested with trypsin enzyme. The peptide molecular mass, obtained by LC MS/MS, were then used to identify the predicted proteins using MASCOT search.

Electrophoretic blotting and immunoblotting: Electrophoretic blotting was performed following Towbin et al. (1979). The separated proteins were transferred onto PVDF membranes using a wet tank-blotting chamber (Mini Trans-blot Cell) from Bio-Rad (Hercules, CA, USA) for 2 hours at a constant current (250 mA). For immunoblotting, the non-specific binding sites on the blots were blocked with 5% dry nonfat milk in Tris-Tween-buffered saline (TTBS: 25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) and incubated with anti-phosphotyrosine (clone 4G10) monoclonal antibody (dilution 1:250) from Millipore (Billerica, MA, USA.) for 1 hour in TTBS at room temperature. The blots were washed with TTBS 3 times (each time for 5 min) and then incubated with goat anti-mouse IgG (H&L, dilution 1:500) linked to alkaline phosphatase (Bio-Rad, Hercules, CA, USA) for 1 hour. After three washes with TTBS (each time for 5 min), the color reaction with NBT/ BCIP (Bio-Rad, Hercules, CA, USA) enabled visualization of the

proteins.

Immunocytochemistry of spermatozoa: The spermatozoa samples were removed from the seminal plasma by centrifugation at 800 *g* for 5 min. The pellets were washed and re-suspended with PBS. A small drop of spermatozoa suspension was smeared on a glass slide, air-dried and fixed in 2% formaldehyde for 1 hour. Following fixation, spermatozoa were permeabilized with 1% Triton X-100 in PBS for 1 hour and non-specific reactions blocked with 5% Normal Horse Serum (Zymed, South San Francisco, CA, USA) in PBS for 30 min at room temperature. To label, the anti-phosphotyrosine (clone 4G10) monoclonal antibody (dilution 1:125) from Millipore (Billerica, MA, USA.) was added to the spermatozoa sample, incubated overnight, and then incubated with peroxidase-conjugated goat anti-mouse antibody (dilution 1:300) from Zymed (South San Francisco, CA, USA) for 1 hour. Color reaction with DAB enabled visualization of the proteins. Observations and photographs were made with a light microscope. In the controls, the incubation of spermatozoa with primary antibody was omitted and the procedure was followed as previously described.

Results

2-DE and LC MS/MS: A total spermatozoa extract protein of 340 µg was assayed by 2-DE and stained with colloidal CBB, and more than 600 protein spots with an isoelectric point (pI) of 3-10, and a relative molecular mass (MW) of 10–97 kDa could be detected (Fig 1). Apart from the new finding of Tektin-4 protein (Thepparat et al., 2012), the markedly different expression of the protein spot at MW 32 kDa attracted our attention. The 32 kDa protein had two distinct patterns among the nine experimental bulls. Pattern A, observed in 6 of the 9 bulls, showed a relatively high protein content of spot Y_1 (32 kDa, pI 5.3). Pattern B, observed in the remaining 3 bulls, expressed two small spots for Y_1 and Y_2 (32 kDa, pI 5.3 and 31.5 kDa, pI 5.5, respectively, Fig 1). According to LC-MS/MS analysis (Table 1), spots Y_1 and Y_2 were both identified as acrosomal protein, the predicted: proacrosin binding protein sp32 in *Bos taurus*. The amino acid sequence coverage of protein spots Y_1 and Y_2 (Fig 2) corresponded to the carboxyl-terminal half of the predicted proacrosin binding protein sp32 found in *Bos taurus* (gi | 194666681).

Immunoblotting and immunocytochemistry: Immunoblotting and immunocytochemistry with anti-phosphotyrosine antibody were investigated to assess whether spot Y_2 (31.5 kDa, pI 5.5) could be p32 (phosphorylated form of sp32), as reported in porcine spermatozoa (Dube et al., 2005). The results of immunoblotting revealed the phosphotyrosine protein spots at approximately MW (kDa)/pI value of 36/8.7-9.6 and 85-97/4.3-6.8 (Fig 3). However, the MW/pI was not in accordance to the spot Y_2 (31.5 kDa, pI 5.5).

According to the identification of spot Y_2 as sp32 (with previous assumption as p32), an acrosomal protein, it should localized on the acrosomal region. The immunocytochemistry of spermatozoa from the bull with pattern B showed the phosphotyrosine proteins labelled with anti-phosphotyrosine antibody at the equatorial segment (Fig 4), however, they were not at the acrosomal region.

Table 1 Identification of protein spot Y_1 and Y_2 by using LC MS/MS

Spot name	Protein name	NCBI accession no.	Species	Score	% Sequence coverage	MW/pI determined	MW/pI calculated
Y_1 (pattern A)	PREDICTED: Proacrosin Binding Protein sp32	gi | 194666681	*Bos taurus*	91	3	32/5.3	62.3/5.1
Y_1 (pattern B)	PREDICTED: Proacrosin Binding Protein sp32	gi | 194666681	*Bos taurus*	169	8	32/5.3	62.3/5.1
Y_2 (pattern B)	PREDICTED: Proacrosin Binding Protein sp32	gi | 194666681	*Bos taurus*	92	6	31.5/5.5	62.3/5.1

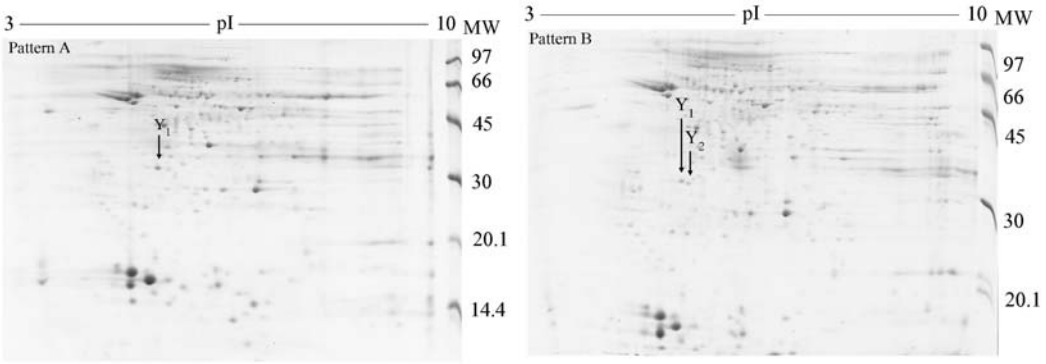

Figure 1 2D-gel of spermatozoa proteins from bulls with two different expression patterns (A and B) at 32 kDa. Location of spots Y_1 (32 kDa, pI 5.3) and Y_2 (31.5 kDa, pI 5.5) are indicated with an arrow. 340 µg of protein was electro-focused in 24 cm dry strips gel with pH range of 3-10. SDS-PAGE was conducted in a 13.5% acrylamide gel plate. The MW standard used ranged between 14.4 and 97 kDa. The MW is provided on the *Y*-axis and pI (3–10) on the *X*-axis of this picture. The colloidal CBB was used for protein staining.

I: Matched peptides of spot Y_1, pattern A

1	MRHLAAGSFL	SLLRVLLLPL	APAPAQDSAS	TSTPGSPLSP	TEYERFFALL
51	TPTWKAETTC	RLRATHGCRN	PTLVQLDQYE	NHGLVPDGAV	CSDLPYASWF
101	ESFCQFTQYR	CSNHVYYAKR	VRCSQPVSIL	SPNTLKEVDS	SPEVPPPTTA
151	TSPKSSHVTG	GKGARNTATT	XXXXXXXXXX	XXXXXXXLSL	GGQEQGQEPK
201	QEQGQEHKQE	RGQEHKQEEG	QEQEEQEEEQ	EEEKQEGQGT	EEALESVSRL
251	QADPEPKFRS	ELVSSNPFSF	TPRVREVEST	PMLMENLQEL	IRSAQEMNEM
301	NDVYDGETIW	RSQSPGSLLQ	LPHVEALLTL	CYSIVENTCV	ITPTAKAWQY
351	LENEILGFGI	SVCDSLGRRH	**LAACTLCDFC**	**SLK**LEQCHSE	ANLQRQQCDS
401	SHKTPFVSPL	LASQSMTIGT	QIGSLKSGRF	YGLDLYGGLR	**MDFWCAR**LAT
451	KGCEDNRVAS	WLQTEFLSFQ	DGDFPTRICD	TEYVQYPNYC	AFKSQQCMMR
501	NRDRKVSRMR	CLQNETYTVL	TPDKSEDLVL	RWSQEFSTLT	LGQAG

II: Matched peptides of spot Y_1, pattern B

1	MRHLAAGSFL	SLLRVLLLPL	APAPAQDSAS	TSTPGSPLSP	TEYERFFALL
51	TPTWKAETTC	RLRATHGCRN	PTLVQLDQYE	NHGLVPDGAV	CSDLPYASWF
101	ESFCQFTQYR	CSNHVYYAKR	VRCSQPVSIL	SPNTLKEVDS	SPEVPPPTTA
151	TSPKSSHVTG	GKGARNTATT	XXXXXXXXXX	XXXXXXXLSL	GGQEQGQEPK
201	QEQGQEHKQE	RGQEHKQEEG	QEQEEQEEEQ	EEEKQEGQGT	EEALESVSRL
251	QADPEPKFRS	ELVSSNPFSF	TPRVREVEST	PMLMENLQEL	IRSAQEMNEM
301	NDVYDGETIW	RSQSPGSLLQ	LPHVEALLTL	CYSIVENTCV	ITPTAKAWQY
351	LENEILGFGI	SVCDSLGRR**H**	**LAACTLCDFC**	**SLK**LEQCHSE	ANLQRQQCDS
401	SHKTPFVSPL	LASQSMTIGT	QIGSLKSGR**F**	**YGLDLYGGLR**	**MDFWCAR**LAT
451	KGCEDNRVAS	WLQTEFLSFQ	DGDFPTR**ICD**	**TEYVQYPNYC**	**AFK**SQQCMMR
501	NRDRKVSRMR	CLQNETYTVL	TPDKSEDLVL	RWSQEFSTLT	LGQAG

III: Matched peptides of spot Y_2, pattern B

1	MRHLAAGSFL	SLLRVLLLPL	APAPAQDSAS	TSTPGSPLSP	TEYERFFALL
51	TPTWKAETTC	RLRATHGCRN	PTLVQLDQYE	NHGLVPDGAV	CSDLPYASWF
101	ESFCQFTQYR	CSNHVYYAKR	VRCSQPVSIL	SPNTLKEVDS	SPEVPPPTTA
151	TSPKSSHVTG	GKGARNTATT	XXXXXXXXXX	XXXXXXXLSL	GGQEQGQEPK
201	QEQGQEHKQE	RGQEHKQEEG	QEQEEQEEEQ	EEEKQEGQGT	EEALESVSRL
251	QADPEPKFRS	ELVSSNPFSF	TPRVREVEST	PMLMENLQEL	IRSAQEMNEM
301	NDVYDGETIW	RSQSPGSLLQ	LPHVEALLTL	CYSIVENTCV	ITPTAKAWQY
351	LENEILGFGI	SVCDSLGRRH	LAACTLCDFC	SLKLEQCHSE	ANLQRQQCDS
401	SHKTPFVSPL	LASQSMTIGT	QIGSLKSGR**F**	**YGLDLYGGLR**	**MDFWCAR**LAT
451	KGCEDNRVAS	WLQTEFLSFQ	DGDFPTR**ICD**	**TEYVQYPNYC**	**AFK**SQQCMMR
501	NRDRKVSRMR	CLQNETYTVL	TPDKSEDLVL	RWSQEFSTLT	LGQAG

Figure 2 Amino acid sequence of the PREDICTED: Proacrosin Binding Protein sp32 in *Bos taurus* (I, II, and III) (http://www.matrixscience.com/). Amino acids are represented by standard single-letter codes. Amino acids in bold represent the matched peptides sequenced by LC MS/MS.

Discussion

The investigation of bovine spermatozoa proteins by using proteomic technologies, in this study, revealed the markedly different expression of sp32. The identification of the 32 kDa, pI 5.3 (spot Y_1) and 31.5 kDa, pI 5.5 (spot Y_2) proteins was the predicted proacrosin binding protein sp32 (*Bos taurus*) at 62.3 kDa, pI 5.1 (Table 1); however, the determined and calculated molecular weight was inconsistent.

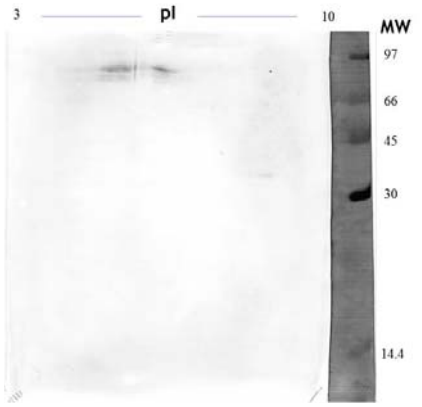

Figure 3 Immunoblotting of bovine spermatozoa proteins labeled with the anti-phosphotyrosine antibody.

'Predicted' signifies that it was derived from a genomic sequence (NW 001494990) as it was not directly sequenced. Thus, the current study is the first report of amino acid sequencing of sp32 at 32 kDa. It has been previously reported that sp32 (32 kDa) in hogs is initially synthesized as a 61 kDa precursor protein and the mature sp32 is the carboxyl-terminal half of the precursor molecule (Baba et al., 1994). It has therefore been suggested that sp32 is produced via a post-translational modification (Baba et al., 1994). We thus hypothesized that the mature bovine sp32 in our study could also be 32 kDa in relative molecular mass, and that the predicted sp32 (62.3 kDa, pI 5.1) was the precursor of sp32. More evidence to support our hypothesis was that the amino acid sequence coverage matched the carboxyl-terminal half of the predicted sp32 (Fig 2). sp32 is a protein located on the acrosomal region of the spermatozoa (Dube et al., 2005). Functionally, sp32 is involved in capacitation and acrosome reaction of spermatozoa (Moos et al., 1993; Baba et al., 1994).

The significant appearance of two small spots of sp32 on 2D gels of three experimental bulls was the main focus of the present study. These are reminiscent of a report on a tyrosine-phosphorylated protein in porcine spermatozoa (p32) that is related to capacitation (Tardif et al., 2001; Tardif et al., 2003; Dube et al., 2005). We further hypothesized that the additional spot (sp32 at spot Y_2; 31.5 kDa, pI 5.5) that appeared in our study could be a tyrosine-phosphorylated form of sp32 (p32), and that this might be due to spontaneous capacitation.

Immunoblotting with anti-phosphotyrosine antibody was then investigated to assess whether spot Y_2 could be p32, as reported in porcine spermatozoa. Unfortunately, immunoblotting with anti-phosphotyrosine antibody did not reveal the phosphotyrosine proteins at spot Y_2 (31.5 kDa, pI 5.5) on the PVDF membrane (Fig 3). Moreover, the results of immunocytochemistry did not localize the phosphotyrosine proteins in the acrosomal region of spermatozoa from the bull with two small spots of sp32 (Fig 4). The current study thus confirmed that spot Y_2 was not a phospho-related protein and that it was not a p32 protein that had undergone spontaneous capacitation. Further characterization and functional analysis of these protein patterns are needed.

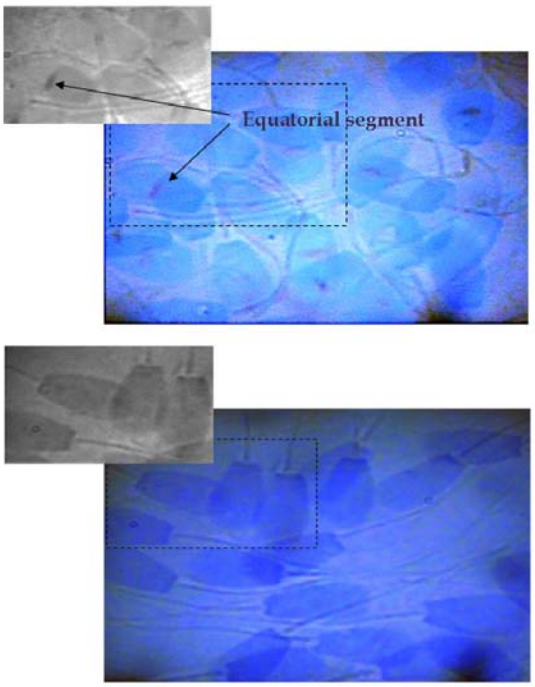

Figure 4 Immunocytochemistry of the anti-phosphotyrosine label in bovine spermatozoa cell. Results showed labeling of anti-phosphotyrosine on the equatorial segment in spermatozoa of bull with pattern B (above) compared to the unlabeled control (below).

Although the results of immunoblotting and immunocytochemistry with anti-phosphotyrosine antibody did not reveal the significant function of sp32, these provide the additional information relating to the proteins of bovine spermatozoa cell. (This seems to be over-claimed in my view. It was not proven in the present study). Further studies are required to investigate the function of the sp32 protein in fertilization process.

In conclusion, our study discovered the mature bovine sp32 with a relative molecular mass of ~32 kDa in two different expression patterns. We also investigated the markedly different expression of sp32 as post-translational modification in terms of phosphorylation. The protein observed at 32 kDa cannot, however, be confirmed as phosphotyrosine protein (p32). This is an intriguing observation inviting further exploration of bovine spermatozoa proteins.

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