รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาโปรตีนในเซมินอลพลาสมาที่สัมพันธ์ถึงความสมบูรณ์พันธุ์ของพ่อแพะ

The study on caprine seminal plasma proteins associated with male fertility

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาและจำแนกชนิดของโปรตีนในเซมินอลพลาสมาของพ่อแพะที่มีการแสดงออกที่แตกต่างกัน และ/หรือโปรตีนที่มีความสัมพันธ์กับความสมบูรณ์พันธุ์ ทำการรีดเก็บน้ำเชื้อจากพ่อแพะอายุระหว่าง 2-5 ปี จำนวน 20 ตัว นำมาตรวจคุณภาพของน้ำเชื้อเพื่อเก็บข้อมูลการเคลื่อนที่ของตัวอสุจิและรูปร่างของตัวอสุจิ น้ำเชื้ออีกส่วนหนึ่งนำไปปั่นเหวี่ยงเพื่อแยกเอาเซมินอลพลาสมาไปแยกโปรตีนด้วยเทคนิค 2D-PAGE แล้วใช้สี Coomassie Brilliant blue ย้อมแผ่นเจล โดยทำตัวอย่างละ 3 ซ้ำ โปรตีนจุดที่ถูกวิเคราะห์ว่ามีปริมาณการแสดงออกแตกต่างกันระหว่างพ่อแพะทดลอง หรือพบมีความสัมพันธ์กับความสมบูรณ์พันธุ์ ถูกนำไปวิเคราะห์หาชนิดของโปรตีนโดยใช้เทคนิค LC MS/MS ข้อมูลอัตราการตั้งท้องที่ใช้น้ำเชื้อแช่แข็งจากพ่อแพะแต่ละตัวไปผสมเทียม ถูกนำมาวิเคราะห์โดยใช้โมเดลทางสถิติเพื่อหาปัจจัยที่มีผลต่ออัตราการตั้งท้อง ได้แก่ โปรตีนที่แสดงออกแตกต่างกัน จำนวนครั้งในการผสม ลำดับครอกของแม่แพะ การแสดงการเป็นสัด คะแนนความสมบูรณ์ของร่างกายแม่แพะ ฤดูกาล และเจ้าหน้าที่ผู้ทำการผสมเทียม ซึ่งข้อมูลนี้ได้รับการสนับสนุนจากศูนย์วิจัยการผสมเทียมและเทคโนโลยีชีวภาพ กรมปศุสัตว์ ผลการศึกษาพบว่า คุณภาพน้ำเชื้อของพ่อแพะทดลองมีคุณภาพดี ซึ่งมีค่าการเคลื่อนที่และรูปร่างของตัวอสุจิปกติสูงกว่า 70 เปอร์เซ็นต์ อัตราการตั้งท้องมีค่าเฉลี่ยเท่ากับ 47.95 เปอร์เซ็นต์ ผลการแยกโปรตีนด้วยเทคนิค 2D-PAGE พบโปรตีนมากกว่า 213 จุด ที่มีค่า pI และ M_r ระหว่าง 3-10 และ 10-97 kDa ตามลำดับ และพบว่ามีโปรตีนที่มีปริมาณมากปรากฏบนแผ่นเจลในช่วง M_r ระหว่าง 10-16 kDa ผลจากการจำแนกพบว่า คือ โปรตีน PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, และ PREDICTED: caltrin-like ที่จำเพาะกับ *Capra hircus* การศึกษาครั้งนี้พบสิ่งที่น่าสนใจคือ โปรตีน PREDICTED: seminal plasma protein PDC 109-like มีการแสดงออกที่แตกต่างกัน 4 รูปแบบ ซึ่งปรากฏแตกต่างกันในระหว่างพ่อแพะแต่ละตัว อย่างไรก็ตาม จากผลการวิเคราะห์ด้วยโมเดลทางสถิติพบว่า การแสดงออกที่แตกต่างกันของโปรตีนชนิดนี้ไม่มีอิทธิพลต่ออัตราการตั้งท้อง ซึ่งไม่เหมือนกับปัจจัยอื่นๆ นอกจากนี้ยังพบว่า โปรตีน PREDICTED: caltrin-like (pI/ Mr เท่ากับ 10/ 10 kDa) มีและไม่มีปรากฏบนแผ่นเจลแตกต่างกันในพ่อแพะแต่ละตัว แต่เนื่องจากโปรตีนชนิดนี้มีค่า pI ประมาณ 10 ซึ่งเป็นช่วงที่เป็นข้อจำกัดในการแยกโปรตีนด้วยเทคนิค 2D-PAGE จึงได้ทำการนำตัวอย่างของเซมินอลพลาสมาของพ่อแพะทดลองทั้งหมด ไปแยกด้วยเทคนิค SDS-PAGE และจำแนก

ด้วยเทคนิค LC MS/MS เพื่อยืนยันผลอีกครั้งหนึ่ง ผลการศึกษาพบว่า แถบโปรตีนขนาด 10 kDa ปรากฏในปริมาณที่แตกต่างกันบนเจลของตัวอย่างเซมินอลพลาสมาจากแพะแต่ละตัว และผลจากการจำแนกโปรตีนแถบนี้พบว่า เป็นชิ้นส่วนเปปไทด์ของโปรตีน PREDICTED: caltrin-like แต่อย่างไรก็ตาม ผลที่ได้จากการแยกโปรตีนด้วย 2 เทคนิคนี้ไม่เป็นไปในทำนองเดียวกัน จึงเป็นเรื่องที่น่าสนใจศึกษาต่อไปด้วยเทคนิค Western blot ผลจากการศึกษาครั้งนี้สรุปได้ว่า โปรตีนที่พบปริมาณมากใน เซมินอลพลาสมาของพ่อแพะ คือ PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, และ PREDICTED: caltrin-like การแสดงออกที่แตกต่างกันของโปรตีน PREDICTED: seminal plasma protein PDC 109-like ทั้ง 4 รูปแบบ ไม่มีผลต่อความสมบูรณ์พันธุ์ของพ่อแพะ

คำสำคัญ: โปรตีนในเซมินอลพลาสมาของแพะ, MS, 2D-PAGE

Abstract

The objective of this study was to investigate the proteins in caprine seminal plasma and identify one(s) that differently expressed and/or associated with male fertility. Semen samples from 20 experimental bucks, 2-5 years of age, were assessed for semen quality in terms of spermatozoa motility and morphology. The aliquot of semen samples from each buck was centrifuged for collecting the seminal plasma. The seminal plasma samples were separated using 2D-PAGE followed by staining with Coomassie Brilliant blue. At least three replicate gels were performed for each sample. The proteins that expressed differently and/or related with pregnancy rate were cut from the gels and identified by LC MS/MS. The history pregnancy data using frozen semen insemination of each buck, from the years 2012 to 2017, were provided by Artificial Insemination and Biotechnology Research Center, Department of Livestock Development, Thailand. The multiple logistic regression analysis was used to estimate the effect of different protein expression and other factors; number of insemination, parity of the does, estrus, body condition score, season, and AI technician on pregnancy rate. The results of semen quality assessment of 20 experimental bucks showed good-quality semen which classified as >70% spermatozoa motility and >70% normal spermatozoa morphology. For fertility, in term of pregnancy rate, the average of pregnancy rate of the experimental bucks was 47.95 percent. The results obtained from 2D-PAGE showed more than 213 protein spots with a pI of pH 3-10 and M_r of 10-97 kDa could be detected in caprine seminal plasma. Of all these spots, there were proteins at molecular weight approximately 10-16 kDa expressed in a large amount on the gel. Identification of these protein spots matched to PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, and PREDICTED: caltrin-like, in Capra hircus. Interestingly, the 4 different expression patterns of PREDICTED: seminal plasma protein PDC 109-like differently showed among bucks.

However, these protein patterns were not significantly associated with pregnancy rate ($p>0.05$) whereas the other factors had effect ($p<0.05$). In addition, this study also detected the different expression of PREDICTED: caltrin-like protein spot on the 2D gel at the pI 10 and M_r 10 kDa. Some bucks showed the high relative protein content on 2D gels whereas the other was absent. Due to the determined pI of this protein was at 10.0 and the limitation on 2D-PAGE performing to separate the basic proteins, the SDS-PAGE and MS were repeatedly conducted to clarify this point. The results obtained from SDS-PAGE separating showed that the protein band intensity among seminal plasma samples were different expression on 1D-gels especially at approximately 10 kDa. The identification results also showed the peptides abundant of 3 peptide fragments of PREDICTED: caltrin-like varied among bucks. Unfortunately, the results obtained from 2D-PAGE and SDS-PAGE was not consistent. The further western blot studies should be conducted to discovery this mysterious protein. In conclusion, the major proteins in caprine seminal plasma were PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, and PREDICTED: caltrin-like. The PREDICTED: seminal plasma protein PDC 109-like expressed in the 4 different patterns and it was not related to buck fertility.

Keywords: Caprine seminal plasma proteins, MS, 2D-PAGE

Executive Summary

The objective of this study was to investigate the proteins in caprine seminal plasma and identify one(s) that differently expressed and/or associated with male fertility in term of pregnancy rate, using 2D-PAGE and LC MS/MS techniques. When 200 µg of total protein was assayed by 2D-PAGE and stained with colloidal Coomassie Brilliant Blue, more than 213 protein spots with a pI of pH 3-10, and M_r of 10-97 kDa could be detected in seminal plasma of an experimental buck. Of all these spots, there were proteins at molecular weight approximately 10-16 kDa expressed in a large amount on the gel. Identification using LC MS/MS technique and MASCOT web-bases search engines found that these protein spots matched to PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, and PREDICTED: caltrin-like, in *Capra hircus*. Interestingly, the results obtained from 2D-PAGE showed the 4 different expression patterns of PREDICTED: seminal plasma protein PDC 109-like with differed among bucks. However, these proteins patterns were not associated with pregnancy rate ($p>0.05$). In addition, this study also detected the different expression of PREDICTED: caltrin-like protein spot on the 2D gel of seminal plasma samples at the determined pI 10 and molecular weight 10 kDa. Some bucks showed the high relative protein content of this protein spot on 2D gels whereas the other was absent. Due to the determined pI of this protein was at 10.0 and the limitation on 2D-PAGE performing to separate the basic proteins, the SDS-PAGE and LC MS/MS were repeatedly conducted to clarify this point. The results obtained from SDS-PAGE separating showed that the protein band intensity among seminal plasma samples were different expression on 1D-gels especially at approximately 10 kDa. The identification results also showed the peptides abundant of 3 fragments of PREDICTED: caltrin-like varied among bucks. Unfortunately, the results obtained from 2D-PAGE and

SDS-PAGE was inconsistent. The further western blot studies should be conducted to discovery this mysterious protein.

The present study, however, is the first study to report on the different expression patterns of PREDICTED: seminal plasma protein PDC 109-like in caprine seminal plasma, using 2D-PAGE and MS technique, although it was not associated with buck fertility.

Introduction

Buck fertility is an important factor in goat reproduction. Several attempts have been done to improve the buck fertility. Strategies including breeding program, hormonal manipulation, and nutritional management were frequently employed. However, the underlying mechanism of fertility involved particularly with seminal plasma proteins would be a straightforward approach to improve buck fertility.

Seminal plasma is the fluid mainly secreted from accessory sex glands. The important of seminal plasma is that it contains the potential proteins which affecting male fertility. Some of seminal plasma proteins bind to ejaculated spermatozoa membrane and make spermatozoa to acquire the fertilization capacity with female gamete. For instance, the heparin binding proteins (HBPs) are the other name of bovine seminal plasma proteins (BSP proteins) and largely secreted from the seminal vesicles (Manjunath *et al*., 1994). These proteins bind to spermatozoa membrane at ejaculation (Manjunath *et al*., 1994) and participate in the process of capacitation (Therien *et al*., 1995). Parallel with advance in proteomic technologies, seminal plasma proteins have been studied in various species such as bull and boar and reported to be the potential markers for male fertility (Killian *et al*., 1993, Roncoletta *et al*., 2006, Sprott *et al*., 2006, Novak *et al*., 2010). Fertility associated antigen (FAA), or BSP-30 kDa, is the good example for such seminal plasma proteins which predominantly found in the higher-fertility bulls (Sprott *et al*., 2006).

In caprine, although many reports have been studied on the seminal plasma proteins (Falci *et al*., 2002, Villemure *et al*., 2003, Rucker *et al*., 2010), however, a few study reported the relation to male fertility. The study on seminal plasma proteins associated with male fertility, by using proteomic techniques such as 2D-PAGE and MS, may discover the protein markers screening buck fertility which will be valuable applications in goat

reproduction improvement. Furthermore, the separating of caprine seminal plasma proteins may provide the information of their mechanism and function effecting on male fertility.

Literature reviews

Proteomic techniques

Two-dimension polyacrylamide gel electrophoresis or 2D-PAGE

2D-PAGE is a powerful method and widely used for analysis the protein complex mixture from cell, tissue, or other biological samples. The principles and methods of 2D-PAGE has been described by Gorg (2004) that this technique separates proteins based on mass and charge by achieve the separation of several thousand different proteins in one gel. The first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI). The second dimension step, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (M_r, relative molecular mass). The most common proteins are separated by isoelectric point in the horizontal direction and by size in the vertical direction. Each spot on the resulting 2D-gel potentially corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained.

The protein profiles which produced by 2D-PAGE can be assessed by image analysis software such as ImageMasterTM 2D Platinum software. The interest protein spot(s) are then identify for it(s) characterization mostly by Mass spectrometry (MS). MS for protein identification relies on the digestion of protein samples into peptides by a sequence-specific protease such as trypsin (de Hoog and Mann, 2004). After the proteins are digested, the peptides are often delivered to a mass spectrometer for analysis via high

performance liquid chromatography (HPLC, LC) separation. HPLC can separate proteins or peptides on the basis of a number of unique or species-specific properties such as charge, size, hydrophobicity and presence of a specific tag or amino acid(s) (Oliva *et al*., 2009). So that, the combines of LC with MS has become a powerful technology for the characterization and identification of peptides and proteins in a complex mixture (Chen *et al*., 2007). According to Brewis and Gadella (2010), LC can be used to separate the trypsinized peptides in both of individual protein spot cut from 2D-gel and global proteins.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis or SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis or SDS-PAGE is a simple and inexpensive method developed by Laemmli (1970). It is a widely used tool for resolving proteins in complex mixtures according to theirs molecular mass. The proteins are denatured with SDS and ß-mercaptoethanol and coated with negative charges of SDS which allows the proteins to migrate in an electric field and separate according to mass/size. The Coomassie Blue is used to visualize all the protein bands on the gel.

Mass spectrometry or MS

MS consists of a few simple modules: an ionization mechanism (a section to separate, select, and fragment peptides), and a detector (de Hoog and Mann, 2004). Cho (2007) has described the method of MS in the following states. A mass spectrometer separates proteins and other analyzes according to their mass-to-charge (m/z) ratio. The molecule is ionized and the ion is propelled into a mass analyzer by an electric field that resolves each ion according to its m/z ratio (ionization of proteins allows them to be propelled towards the analyzer by virtue of charge repulsion). Then the detector passes the information to the computer for analysis.

Frequency used ionization methods include electro-spray ionization (ESI), and matrix-assisted laser desorption/ ionization (MALDI), and surface-enhanced laser desorption/ ionization (SELDI), because they cause little or no fragmentation of the molecule during the ionization and desorption process.

The resulting peptide mass fingerprints are then searched against theoretical fingerprints of sequences in the databases to arrive at protein identifications. Also, a given peptide map may not have sufficient information to identify the protein or the protein may not be present in the database. In such case, the proteins may be analyzed by tandem mass spectrometry (MS/MS) (Dhingra *et al*., 2005). This sequence data is then used to search existing protein databases to achieve a match and therefore the protein identification (Brewis and Gadella, 2010).

Fertility associated seminal plasma proteins in bull and boar

There were many reports on the fertility associated seminal plasma proteins in the bull. Killian *et al*. (1993) were undertaken to determine whether bovine seminal plasma contained protein markers associated with bull fertility indicated by percentage point deviation of AI cow non-return rate values, and whether these markers were of value in predicting bull fertility. Two-dimension polyacrylamide gel electroporesis (2D-PAGE) of seminal plasma sample which obtained from 35 Holstein bulls that passed breeding soundness examination (BSE) and routinely used in AI centers indicated two proteins (26 kDa, pI 6.2 and 55 kDa, pI 4.5) predominated in higher-fertility bulls while the other two lower molecular proteins (16 kDa, pI 4.1 and 16 kDa, pI 6.7) predominated in lower-fertility bulls. These researchers interested in four seminal plasma proteins that associated with bull fertility and further performed the specific studies on these proteins. Cancel *et al*. (1997) studied to corroborate the relationship between the 55 kDa protein and Holstein bull fertility, and to identify the 55 kDa fertility-associated proteins from bovine seminal plasma. They found that the correlation

coefficient between calculated fertility and actual bull fertility was similar (r=0.87) to those reported by Killian *et al*. (1993) and protein characterization revealed that it was Osteopontin (OPN). The 26 kDa protein was identified as lipocalin-type prostaglandin D syntase (PGDS) (Gerena *et al*., 1998). Moreover, the low-molecular weight antifertility peptide (16 kDa, pI 6.7) was also identified as spermadhesin Z13 (Moura *et al*., 2006).

Furthermore, Moura *et al*. (2006) evaluated the expression of proteins in the accessory sex gland fluid of Holstein dairy bulls and their relationships with fertility indexes (percentage point deviation of their non-return rate). Using 2D-PAGE, they found that the average of 52±5 spots were detected in the accessory sex gland fluid, but there were no spots unique to groups of either high- or low- fertility sires. The former were neither less nor more homogeneous than the latter, based on correlations of all matched spots between pairs of accessory sex gland fluid map. However, high fertility of bulls was significantly associated with lower expression of 14-kDa spermadhesin Z13 isoforms and higher amounts of 55 kDa Osteopontin and 58 kDa phospholipase A_2 (PLA_2) isoforms. The average intensity of 5 spots identified as BSP 30 kDa in the accessory sex gland fluid gels had a quadratic association with fertility indexes (R^2 = 0.18, P = 0.03). Percentage point deviation values of bulls were related (R^2 = 0.56) to the quantity of spermadhesin Z13, Osteopontin, and BSP 30 kDa in the accessory sex gland fluid polypeptide maps. Bull fertility was also determined by another equation (R^2 = 0.53) with spermadhesin Z13, BSP 30 kDa and PLA_2 as independent variables. From that study, Moura *et al*. (2006) concluded that interaction among several proteins in accessory sex gland fluid explain a significant proportion of the variation in fertility scores of Holstein dairy bulls.

Gathering information from the above reviews, it is noticed that Osteopontin and spermadhesin Z13 may be the potential marker to predict Holstein bull fertility because of the consistent results that show Osteopontin

presence with the high fertility bulls and spermadhesin Z13 is prevailed in low fertility bulls.

Additionally, the protein mainly secreted from accessory sex glands is Heparin Binding Proteins (HBPs). It binds to sperm membrane at ejaculation (Miller *et al*., 1990). The presence of specific HBPs at sperm membrane indicates affinity of sperm to heparin, subsequently the ability of sperm to capacitation and acrosome reaction, and thus the fertility potential of a bull. There were many reports on the relationship between the ejaculated sperm membrane HBPs and the fertility of the range beef bulls and AI dairy bulls (percentage of cows pregnant of total number of palpated cows, non-return rate). Sperm from high-fertility Holstein bulls have a greater binding affinity for heparin than sperm from less-fertile bulls (Marks and Ax., 1985). The range beef bulls (Red Angus, Santa Gertrudis, Gelbvieh, and Santa Gertrudis x Gelbvieh) with increased fertility possessed HBP with the greatest affinity for heparin (HBP-B5), not just its present, in sperm membrane (Bellin *et al*., 1994). Western blots using monoclonal antibody were used to detect the presence of individual proteins in sperm membranes of Santa Gertrudis bulls and found that bulls with 30, 24, and 21.5-kDa HBP, or bulls with only the 30-kDa HBP were 40 percentage points more fertile than bulls without those three HBP; bull with the 30-and 21.5-kDa HBP in sperm had intermediate fertility of 61.3% (Bellin *et al*., 1996).

The presence of the single 30-kDa HBP which has been named fertility associated antigen (FAA), in sperm membrane could be used as a marker to indicate fertility potential of the both range beef bulls and AI beef bulls. Groups of range beef bulls (Santa Gertrudis and Santa Cruz breed) with 30-kDa HBP (FAA-positive) in sperm membrane were nine percentage points more fertile than groups of bulls without 30-kDa HBP (FAA-negative) (Bellin *et al*., 1998). The average pregnancy rate to first AI service was 7-9 percentage points higher when using semen from AI beef bulls of mixed breeds (Angus,

Red Angus, Barzona, Brangus, Brahman, Hereford, Polled Hereford Simmental, and Tarentaise) with sperm 30-kDa HBP (Sprott *et al*., 2000). Recently, this protein was routinely used to identify the greater fertility bulls (Ax, 2005).

Another reports on protein secreted mainly from the accessory sex glands that bind to sperm membrane associated with bull fertility were performed by Roncoletta *et al*. (2006). They found that 27 spots proteins of sperm membranes were prevalent in the higher fertility (pregnancy rates > 80%) Nelore AI bulls (*Bos taurus indicus*) and just one spot (14.8 kDa, pI 4.4) was prevalent in the lower fertility group (pregnancy rates < 68%). Spot 14.8 kDa, pI 4.4 was identified as bovine seminal plasma A3 (BSP-A3). Only one spot (15 kDa, pI 4.2) of 27 proteins that prevailed in higher fertility bulls was also identified as acidic seminal fluid protein (aSFP). Furthermore, aSFP in seminal plasma fluid was reported as semen freezability marker of bulls (Jobim *et al*., 2004).

All evidences described above indicated that proteins present in the seminal plasma influenced on bull fertility and appear to be a value in predicting the difference in relative fertility among bulls.

In porcine, seminal plasma proteins including major seminal plasma glycoprotein (PSP-I) and glutathione peroxidase (GPX5) have been reported to be the fertility markers by Novak *et al*. (2010). They found that total piglets born was negatively correlated with PSP-I ($r = -0.76$, $p = 0.01$) and fertility index and farrowing rate tend to be positively correlated ($p<0.01$) with GPX5. In addition, Flowers *et al*. (2001) reported that the 26 kDa, pI 6.2 and 55 kDa, pI 4.8 proteins were predominant in seminal plasma of boar with high fertility: high farrowing rate (more than 86%) and number of piglets born alive (more than 11 piglets).

Investigation of seminal plasma proteins in ram and goat

The major proteins of ram seminal plasma is spermadhesin and other major proteins (RSP-15 kDa, RSP-16 kDa, RSP-22 kDa, and RSP-24 kDa,) belong to binder of spermatozoa proteins family (BSPs) which had the function on capacitation and sperm-egg interaction (Bergeron *et al*., 2005). In addition, the ram seminal plasma proteins had the function involving in cold shock protection and improving the post-thawed sperm motility. The RSVP14 (homolog to RSP-15 kDa) and RSVP20 which secreted from seminal vesicles can protect the spermatozoa against cold shock (Barrios *et al*., 2000 and Fernandez-Juan *et al*., 2006). Bernardini *et al*. (2011) reported that seminal plasma proteins which bound to sperm membrane were conserved among ram breeds and that when added to frozen/ thawed semen (along with an energy source), they repaired ram spermatozoa damage and enhanced sperm motility. Furthermore, there were reported on the relationship between ram seminal plasma proteins and sperm motility (Rodrigues *et al*., 2013).

Though the proteomic characterization of mammalian seminal plasma revealed the divergence in protein composition, however, some protein such zinc alpha glycoprotein (ZAG) was mainly found in ram and buck seminal plasma (Druart *et al*., 2013).

A few studies of seminal plasma proteins of goat have been reported. The isolation and characterization of gelatin-binding proteins from goat seminal plasma indicated that GSP (GSP, goat seminal plasma proteins) homolog to BSP proteins (BSP, bovine seminal plasma proteins) which exist in several forms in each species and possibility play a common biological role (Villemure *et al*., 2003). The goat seminal plasma proteins also be performed with SDS-PAGE and identified using MS. The results showed that the bodhesin-2, belong to a new family of spermadhesins, is the interested major seminal plasma protein. However, the researcher did not carry out the relationship with the buck fertility (Rucker *et al*., 2010). In addition, the goat

seminal plasma proteins, Heparin-affinity proteins (HAPs), are under seasonal control and associated with sperm function during breeding and non-breeding seasons (Falci *et al*., 2002).

The objectives

The objective of this study was to investigate the proteins in caprine seminal plasma and identify one(s) that differently expressed and/or associated with male fertility, pregnancy rate, using 2D-PAGE and LC MS/MS techniques.

Methodology

Experimental animals

The 20 bucks consist of 6 Anglonubian, 11 Boer and 3 Kalahari red breed that maintained at Songkla, Suratthani, and Nakhonratchasima Artificial Insemination and Biotechnology Research Center, Department of Livestock Development, Thailand, were used in this study. The bucks were between 2 to 5 years of age.

Semen and Seminal plasma collection

Semen samples were collected once a week (at least 3 consecutive weeks) by using an artificial vagina. The first ejaculated semen was used in this study. Seminal plasma was obtained from the semen by centrifuging at 800× g for 5 min. The supernatant seminal plasma was then transferred to 1.5 ml tubes and centrifuged at 10,000× g for 60 min at 4°C. The supernatant of each sample was divided into 1.5 ml aliquots and stored at -70°C for further measuring protein concentration.

Semen quality assessment

Semen quality was assessed, based on spermatozoa motility, and spermatozoa morphology, as per the Society for Theriogenology guideline (Chenoweth *et al*., 1993). Individual progressive motility was evaluated by diluting the semen to 50 times with Tris extender and a drop put under a cover slip. At least 300 spermatozoa, selected randomly, were observed for the percentage of motile spermatozoa with forward progression using a microscope at 400× magnification. The morphological evaluation included (a) counting 300 spermatozoa at 1000× magnification with an eosin-nigrosin stained, air dried slide (b) determining the percentage of normal spermatozoa and (c) recording any abnormal morphology, both primary and secondary.

Protein assay

The seminal plasma samples were assayed for protein concentration (Bradford, 1976) using bovine serum albumin as the standard. To perform the standard curve, the 100-µl of BSA series dilutions were made to give final concentrations with 3 replicates of 20, 30, 50, 70 and 90 µg of protein. The 5-ml aliquot of dye reagent was added to each series dilution then thoroughly mixed. The mixture was incubated at room temperature for at least 5 min then absorbance was measured at 595 against the reagent blank within 1 h of mixing.

The linear regression between the absorbance and protein concentration were employed to create the standard equation. In order to measure the protein concentration in the samples, triplicates of each sample were prepared by pipetting a respective 100 µl of 1:100 dilution with de-ionized water into a test tube. The dye reagent with a similar solution for performing the BSA standard was added to each tube (5 ml). After thoroughly mixing, the absorbance (at 595) of the solution was measured within 5 min and 1 h after mixing. The protein content was calculated by

replacing the average absorbance of each sample in a regression equation of the standard curve.

2D-PAGE

Each sample of seminal plasma was thawed at 4°C and prepared on ice before the 2D-PAGE was performed. The 2D-PAGE was performed according to O'Farrell (1975). Briefly, the isoelectric focusing (IEF) was performed in Isoelectric Focusing System in 13 cm IPG dry strips with a pH range 3-10. Prior to the IEF, the seminal plasma sample was diluted to 200 µg of protein in 250 µl of re-hydration solution (7M urea, 2M thiourea, 4% w/v CHAPS, 2% w/v DTT, 0.5% v/v IPG buffer, 0.002% Bromophenol blue). This mixing solution was loaded to the Immobiline DryStrip Reswelling tray, the IPG dry strip was placed with the gel side down and overlayed with Immobiline DryStrip cover fluid. The IPG dry strip was allowed to re-hydrated overnight (10-20 h).

The focusing was performed on an Isoelectric Focusing Unit using the following 4 steps: (i) step and hold 500 V for 500 Vh (ii) gradient up to 1000 V for 800 Vh (iii) gradient up to 8000 V for 11300 Vh and (iv) step and hold 8000 V for 7400 Vh. The focused was equilibrated in a buffer (6 M 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 Iodoacetate (IAA) for 30 min.

After being equilibrated, the strip was done in a second dimension of 12.5% SDS-polyacrylamide gel. The low molecular weight standard, range 14.4-97 kDa, was loaded to the gel. The equilibrated IPG gel strip was embedded in a sealing solution (0.5% agarose in 25 mM Tris-base, 192 mM glysine, 0.1% SDS and 0.002% bromophenol blue).

The vertical setup was used using 25 mA/gel. After that the gel was stained in colloidal Coomassie Brilliant Blue G-250 (0.08% Coomassie Brilliant Blue G-250, 8% ammonium sulfate, 0.8% phosphoric acid, 20% methanol)

overnight. The gel was de-stained with de-ionized water for at least 24 h or until the background was clear. Each gel was scanned with an ImageScanner System and analyzed spots by ImageMasterTM 2D Platinum software (Amersham BioSciences).

SDS-PAGE

The seminal plasma sample was performed using SDS-PAGE based on Laemmli (1970) using a vertical slab gel apparatus with the stacking gel containing 5% acrylamide and the resolving gel with 12.5% acrylamide. In brief, the sample was diluted to 15 µg of protein in 15 µl of reducing buffer (20% glycerol, 1%SDS, 0.125M Tris/HCl, 2% β-mercaptoethanol and 0.5% bromophenol blue), heated for 5 min at 95°C and centrifuged at 8,000 ×g for 10 min. The supernatant was loaded to a single well and the molecular mass marker, range 10-180 kDa, was also used to calibrate the protein mass on the gel. Electrophoresis was subjected at constantly 20 mA/gel. After that the gel was stained in colloidal Coomassie Brilliant Blue G-250 (0.08% Coomassie Brilliant Blue G-250, 8% ammonium sulfate, 0.8% phosphoric acid, 20% methanol) and washed with de-ionized water until the background was clear. The protein pattern of gel was recorded using Image Scanner (Amersham BioSciences).

LC MS/MS

The interesting protein spots/ bands were cut from 2D-/1D-gel and identified using the LC MS/MS technique. Each spot/ band sample was digested with trypsin enzyme then the digested peptides were analyzed by LC/MS/MS mass spectrometry, (Thermo Electron). The LC separation and MS analysis details were: HPLC system, Finnigan SurveyorTM MS pump with a flow splitter; Column, 0.18×100 mm C18 (Thermo Electron) particle size 5µm; Flow rate, 100µl/min; Mobile phase A, water with 0.1% formic acid; Mobile phase B, acetonitrile with 0.1% formic acid; Gradients, 7-60 %B in 30 min

65-80 %B in 5min and hold 5min, 80-7 %B in 2min; Mass Spectrometer, Finnigan LTQ; Ionization mode, NanoSpray, positive ion; Capillary temperature, 200 C; Spray needle voltage, 1.8 KV; Mass range, 400-1,600 m/z; Scan sequence, Full-scan MS, MS/MS scan; Acquisition modes, Normal, Data DependentTM and Dynamic ExclusionTM. The molecular weight values of the trypsinized peptides obtained by LC MS/MS were then used to identify the predicted proteins using MASCOT web-bases search engines.

Fertility data

A total of 11,814 fertility records, pregnancy rate, of 20 experimental bucks were provided by Artificial Insemination and Biotechnology Research Center, Department of Livestock Development, Thailand, based on the breeding using frozen semen insemination. The data was collected during the years 2012 to 2017.

Statistical analysis

The multiple logistic regression analysis was used to estimate the effect of different protein expression and other factors on pregnancy rate. The full statistical model included the effects of number of insemination (1 and 2), parity of the does (0, 1, 2, 3, and ≥4), estrus (induced and natural estrus), body condition score (2, 3, and 4), season (summer, light and heavy rain), AI technician (1, 2, 3, to 64), and protein expression patterns (pattern B, C, and D of PREDICTED: seminal plasma protein PDC 109-like) as shown in the equation below The SAS software Inc (1998) was used to be the tool for statistical analysis.

$$ln\left(\frac{P}{1-P}\right) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 + \varepsilon$$

Where P = probability of pregnancy

α = constant value

β = coefficient of variation

X_1 = number of insemination (1 and 2)

X_2 = parity of the does (0, 1, 2, 3, and ≥4)

X_3 = estrus (induced and natural estrus)

X_4 = body condition score (2, 3, and 4)

X_5 = season (summer, light rain and heavy rain)

X_6 = AI technician (1, 2, 3, to 64)

X_7 = protein expression patterns (B, C, and D)

ε = error

Results

Profiles and identification of major seminal plasma proteins

After performing the protein separating using 2D-PAGE and stained with colloidal Coomassie Brilliant Blue, more than 213 protein spots with a pI of pH 3-10, and M_r of 10-97 kDa could be detected in seminal plasma of an experimental buck (Figure 1). Of all these spots, there were proteins at molecular weight approximately 10-16 kDa expressed in a large amount on the gel (spot a, b, c, d, and e). Identification using LC MS/MS technique and MASCOT web-bases search engines found that the protein spot a, b, c, d, and e matched to PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, and PREDICTED: caltrin-like, respectively, in *Capra hircus* (Table 1).

The 4 different patterns of PREDICTED: seminal plasma protein PDC 109-like

The results obtained from this study clearly showed the 4 different patterns of PREDICTED: seminal plasma protein PDC 109-like (Figure 2). Among 20 experimental bucks screening revealed that all of them had the 3 major spots of PREDICTED: seminal plasma protein PDC 109-like at spot a, b, and c with present or absence the small spot(s) in different degree under spot c. Of all bucks—(1) one had no any spot (pattern A) (2) three had one small spot (spot f, pattern B) (3) and (4) four and twelve had 2 small spots in the different degree (spot g and h, pattern C; and spot I and j, pattern D, respectively)— under spot c. The all small spots were identified as the similar protein as PREDICTED: seminal plasma protein PDC 109-like (Table 2).

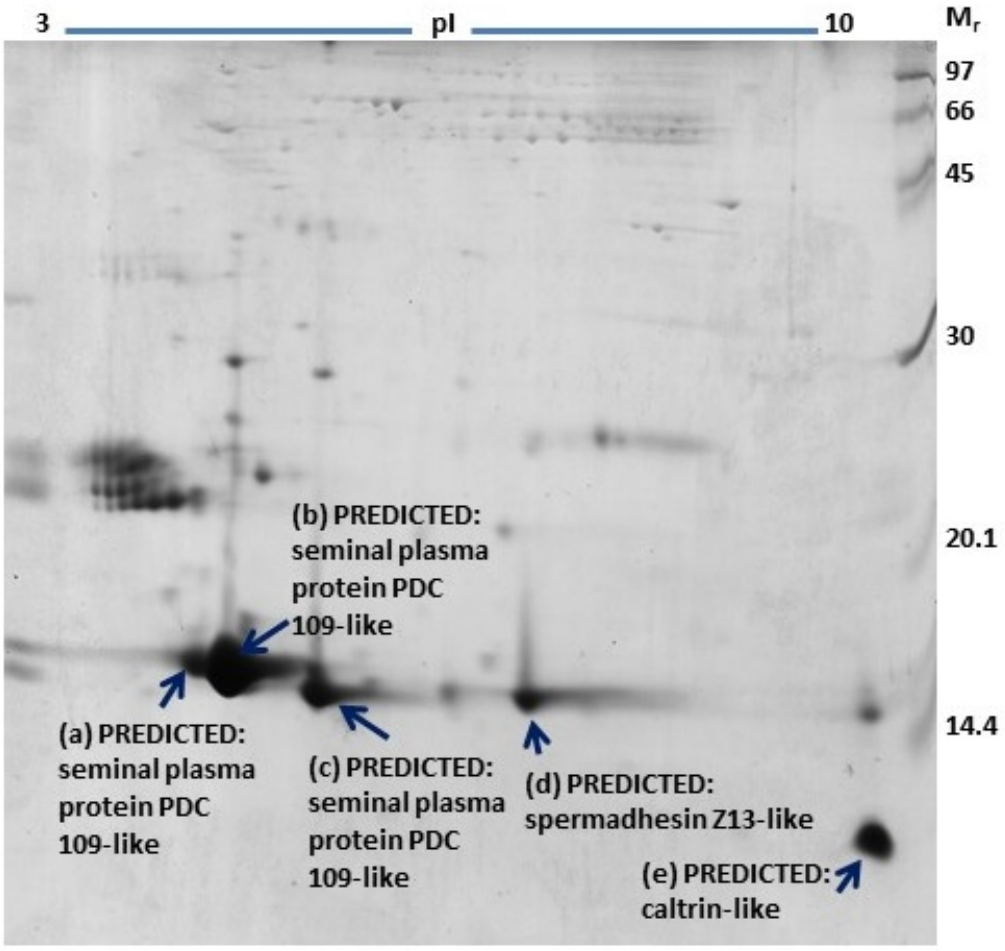

Figure 1 2D-gel of major proteins in caprine seminal plasma. Location of spot a, b, c, d and e are indicated with an arrow. 200 µg of protein was electro-focused on 13 cm DryStrip gel (range, pH 3-10). SDS-PAGE was conducted on a 12.5% acrylamide gel plate. The M_r standard used ranged between 14.4-97 kDa. In this figure, M_r is on the Y-axis and pI (3-10) on the X-axis. Colloidal Coomassie Brilliant Blue G-250 was used for the protein staining.

Table 1 Major proteins in Caprine seminal plasma identified by LC MS/MS

Spot	Protein name	NCBI accession no.	Species	Score	M_r/pI determined	M_r/pI calculated
a	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	143	16.0/4.8	14.64/5.43
b	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	404	16.0/5.1	14.64/5.43
c	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	104	15.0/5.6	14.64/5.43
d	PREDICTED: spermadhesin Z13-like	gi|548523829	*Capra hircus*	217	15.0/7.1	15.34/6.18
e	PREDICTED: caltrin-like	gi|548509324	*Capra hircus*	64	10.0/10.0	8.9/10.21

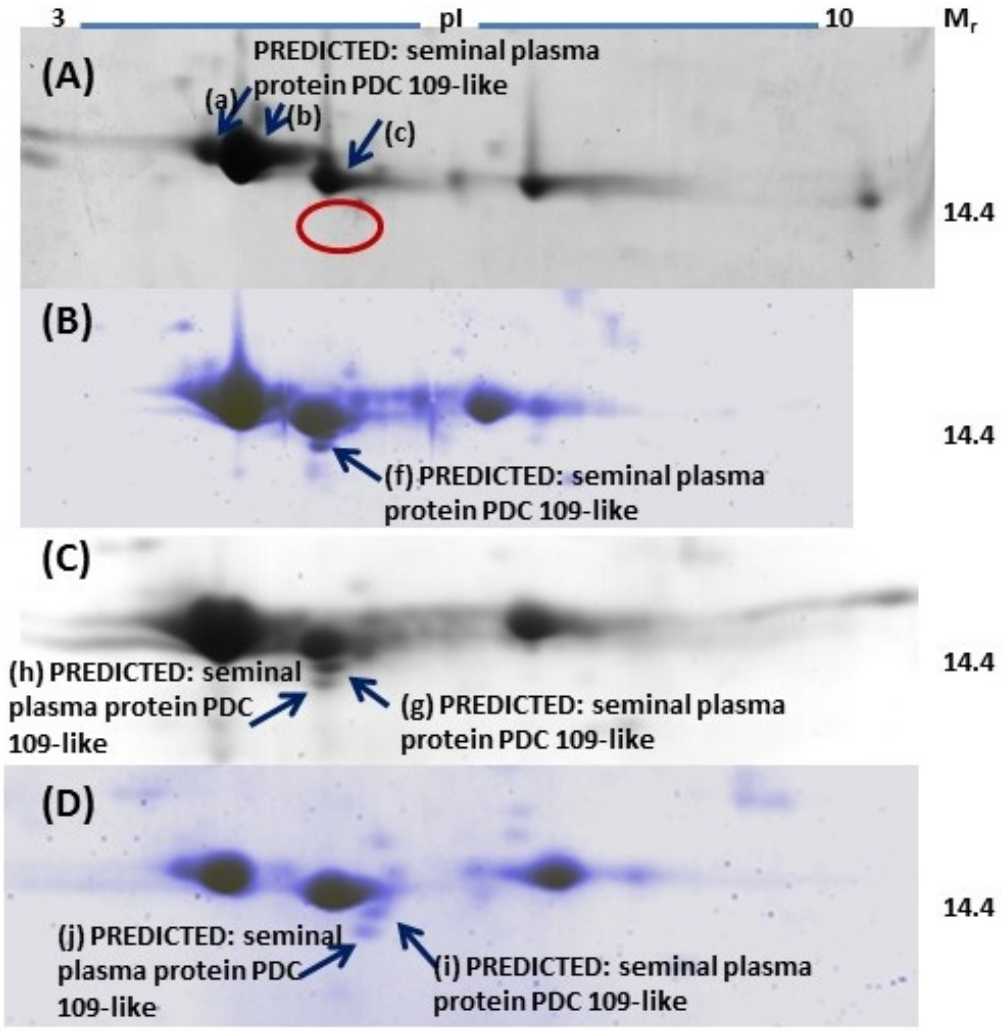

Figure 2 2D-gel of caprine seminal plasma proteins with 4 different expression patterns of PREDICTED: seminal plasma protein PDC 109-like (A, B, C and D). Location of spot f, g, h, i and j are indicated with an arrow. 200 µg of protein was electro-focused on 13 cm DryStrip gel (range, pH 3-10). SDS-PAGE was conducted on a 12.5% acrylamide gel plate. The M_r standard used ranged between 14.4-97 kDa. In this figure, M_r is on the Y-axis and pI (3-10) on the X-axis. Colloidal Coomassie Brilliant Blue G-250 was used for the protein staining.

Table 2 Identification using LC MS/MS of particular protein spots of PREDICTED: seminal plasma protein PDC 109-like in Caprine seminal plasma

Spot	Protein name	NCBI accession no.	Species	Score	M_r/*pI* determined	M_r/*pI* calculated
f	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	45	14.0/5.1	14.64/5.43
g	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	63	14.0/5.1	14.64/5.43
h	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	45	13.0/5.1	14.64/5.43
i	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	118	14.0/5.3	15.08/5.43
j	PREDICTED: seminal plasma protein PDC 109-like	gi|548504897	*Capra hircus*	92	13.0/5.2	15.08/5.43

Different expression of PREDICTED: caltrin-like

PREDICTED: caltrin-like, with a determined pI and M_r of 10.0 and 10.0 kDa, respectively, showed the different expression among the experimental bucks (Figure 3). Some of them showed high relative protein content of this protein spot on 2D-gels whereas the other was absent. Due to the determined pI of PREDICTED: caltrin-like was at 10.0 and the limitation on 2D-PAGE performing to separate the basic proteins, the SDS-PAGE and LC MS/MS were conducted to clarify this protein expression. The results obtained from SDS-PAGE separating showed that the protein band intensity among seminal plasma samples were different expression on 1D-gels especially at approximately 10 kDa, indicated with an arrow, (Figure 4). The 10 kDa protein band of all 20 seminal plasma samples were identified using LC MS/MS and MASCOT web-bases search engines focusing on PREDICTED: caltrin-like. The screening results among 20 experimental bucks showed that three had no match with PREDICTED: caltrin-like whereas six, five, and six had 1, 2, and 3 fragment peptide(s), respectively, matched to this protein at the carboxyl-terminal (Figure 5). The score and percentage of protein sequence coverage of 1, 2, and 3 fragment peptide(s) were 34-82/ 5-6, 94-232/ 12-13, and 175-333/ 17-20, respectively. However, the relative protein content of PREDICTED: caltrin-like spot on 2D-gel (some samples) was inconsistent with the peptides abundant obtained from 1D gel separation. Thus the further western blot studies should be performed to clarify this point.

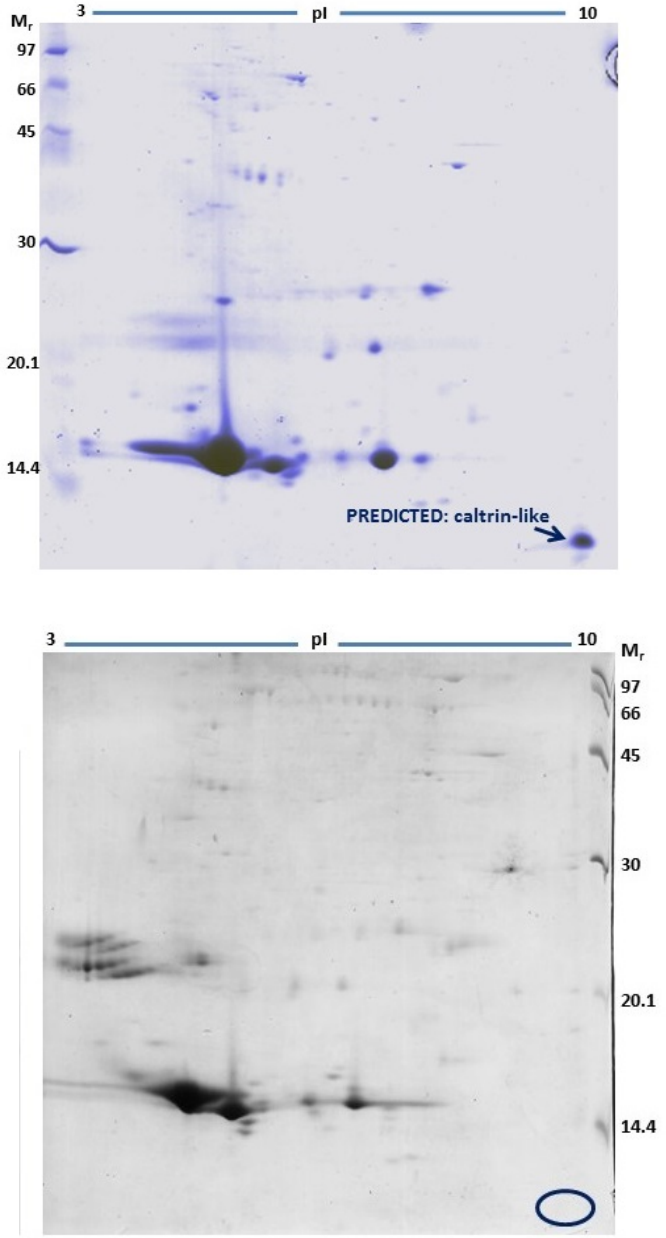

Figure 3 2D-gel of caprine seminal plasma proteins with different expression of PREDICTED: caltrin-like. 200 µg of protein was electro-focused on 13 cm DryStrip gel (range, pH 3-10). SDS-PAGE was conducted on a 12.5% acrylamide gel plate. The M_r standard used ranged between 14.4-97 kDa. In this figure, M_r is on the Y-axis and pI (3-10) on the X-axis. Colloidal Coomassie Brilliant Blue G-250 was used for the protein staining.

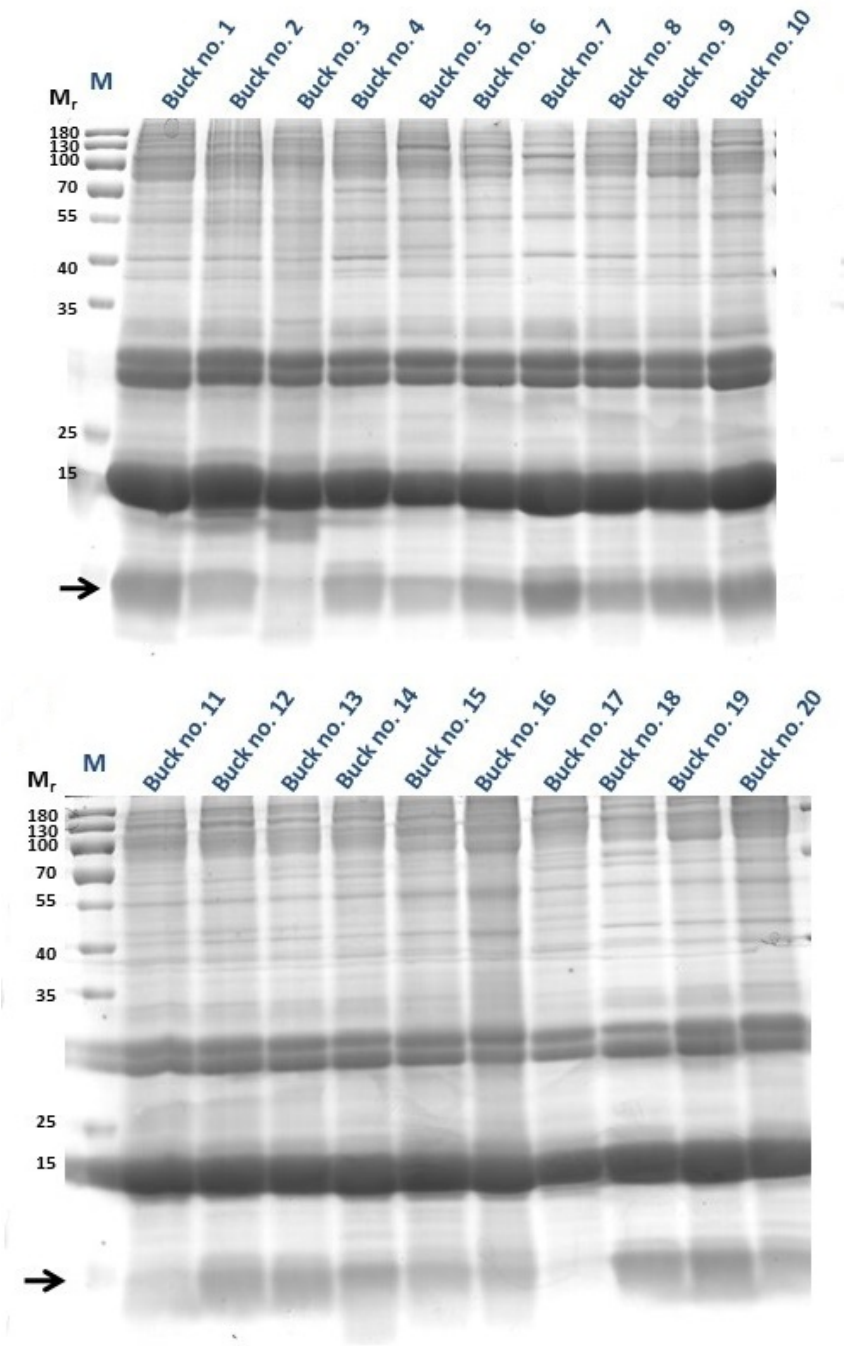

Figure 4 1D-gel of seminal plasma of 20 experimaental bucks. 15 µg of protein was conducted on a 12.5% acrylamide gel plate. The M_r standard was on the first lane ranged between 10-180 kDa. An arrow indicated the aprroximately 10 kDa protein band.

I: Matched peptide of 1 fragment to PREDICTED: caltrin-like

1	MRKLRPEMVQ	CLRTHSQQVA	KPWSGTSPRQ	VAEAWTRWQP	QTPASGAVAL
51	AESSAPLQMM	AGRRSWPAMA	IVLLALLVCL	GELVDADSKP	QPSGEKASRE
101	KHHFSLSHYA	KLANRLLNPK	**LLGSFLSK**RI	GDHANRPVK	

II: Matched peptides of 2 fragments to PREDICTED: caltrin-like

1	MRKLRPEMVQ	CLRTHSQQVA	KPWSGTSPRQ	VAEAWTRWQP	QTPASGAVAL
51	AESSAPLQMM	AGRRSWPAMA	IVLLALLVC	GELVDADSKP	QPSGEKASRE
101	KHHFSLSHYA	KLANRLLNPK	**LLGSFLSKRI**	**GDHANRPVK**	
or 1	MRKLRPEMVQ	CLRTHSQQVA	KPWSGTSPRQ	VAEAWTRWQP	QTPASGAVAL
51	AESSAPLQMM	AGRRSWPAMA	IVLLALLVCL	GELVDADSKP	QPSGEKASRE
101	KHHFSLSHYA	K**LANRLLNPK**	**LLGSFLSKR**I	GDHANRPVK	

III: Matched peptides of 3 fragments to PREDICTED: caltrin-like

1	MRKLRPEMVQ	CLRTHSQQVA	KPWSGTSPRQ	VAEAWTRWQP	QTPASGAVAL
51	AESSAPLQMM	AGRRSWPAMA	IVLLALLVCL	GELVDADSKP	QPSGEKASRE
101	KHHFSLSHYA	K**LANRLLNPK**	**LLGSFLSKRI**	**GDHANRPVK**	

Figure 5 Amino acid sequence of PREDICTED: caltrin-like (http://www.matrixscience.com/). Amino acids are represented by standard single letter codes. Amino acids in bold represent the matched peptide(s) sequence by LC MS/MS.

Semen quality and fertility of experimental bucks

The semen quality assessment of 20 experimental bucks showed good-quality semen which classified as >70% spermatozoa motility and >70% normal spermatozoa morphology.

For fertility, in term of pregnancy rate, the average of pregnancy rate of the experimental bucks was 47.95 percent, using frozen semen insemination.

Factors affecting the pregnancy rate

The statistical analysis results showed that the factors of number of insemination, parity of the does, estrus, body condition score, season, and AI technician had significant effect on the pregnancy rate (Table 3).

According to the function of PREDICTED: seminal plasma protein PDC 109-like involving in sperm capacitation and fertilization (more details in discussion section), the relationship between fertility (pregnancy rate) and these protein expression patterns was statistical analyzed. The results showed that PREDICTED: seminal plasma protein PDC 109-like patterns (pattern B, C, and D) had no influence on the pregnancy rate (Table 3). Pattern A was excluded from the data analysis because there was only one sample.

Table 3 Logistic regression model of risk factors associated with pregnancy rate in goats

Risk factors	β	*SE*	Odd ratio	p-value
Number of insemination				
2	Reference group			
1	-0.5485	0.0933	0.578	<.0001
Parity				
0	Reference group			
1	0.2493	0.074	1.283	0.0008
2	0.3692	0.0731	1.447	<.0001
3	0.4203	0.0767	1.522	<.0001
>4	0.3945	0.0762	1.484	<.0001
Estrus				
Natural	Reference group			
Induced	-0.5044	0.058	0.604	<.0001
Body condition score				
2	Reference group			
3	0.1937	0.0998	1.214	0.0523
4	0.4088	0.1447	1.505	0.0047

Table 3 (cont.)

Risk factors	β	*SE*	Odd ratio	p-value
Season				
Summer	Reference group			
Light rain	0.1270	0.0521	1.135	0.0148
Heavy rain	0.0769	0.0489	1.08	0.1163
AI technician				
1	Reference group			
2	3.3208	1.1516	27.684	0.0039
3	1.5820	0.9035	4.865	0.0799
4	2.0602	0.5858	7.848	0.0004
5	1.6452	0.4519	5.182	0.0003
...				
64	1.2691	1.2691	3.558	0.0020
Patterns of PREDICTED: seminal plasma protein PDC 109-like				
Pattern D	Reference group			
Pattern C	0.0449	0.0528	1.046	0.3946
Pattern B	-0.0973	0.0611	0.973	0.1115

Discussion

The separation of caprine seminal plasma proteins by using 2D-PAGE technique displayed more than 213 spots on the 13 cm strip-2D gel. The expression of major seminal plasma proteins was shown at molecular weight approximately 10-16 kDa which were identified by LC MS/MS technique as PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, and PREDICTED: caltrin-like. The expression of PREDICTED: seminal plasma protein PDC 109-like patterns attracted the attention of this study as they were expressed differently among the 20 experimental bucks even though they did not associated with fertility. Another interesting protein was PREDICTED: caltrin-like which clearly showed presence or absence on 2D gels of each buck. However, this study could not clarify the expression of this protein although SDS-PAGE and LC MS/MS were used to be the additional

assessment tools. Moreover, identification of these major seminal plasmas proteins matched to PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, and PREDICTED: caltrin-like means that they are derived from a genomic sequence (gi|548504897, gi|548523829, and gi|548509324, respectively) not the result directly from amino acid sequence. Thus Identity and functions of these proteins are discussed accordingly.

This study showed the high relative protein content of 3 spots of PREDICTED: seminal plasma protein PDC 109-like at 16.0 kDa, pI 4.8 (spot a), 16.0 kDa, pI 5.1 (spot b), and 15.0 kDa at pI 5.6 (spot c). These proteins may be the Goat Seminal Plasma Proteins (GSP) reported by Villemure *et al*. (2003). They found that four protein bands appeared on 1D gel were GSP-14 kDa, GSP-15 kDa, GSP-20 kDa, and GSP-22 kDa and homologous to Bovine Seminal Plasma Proteins (BSP proteins) which presented in bovine seminal plasma. The 3 major protein spots which found in this study should be the GSP-14 kDa, GSP-15 kDa due to the closely molecular weight expression. However, the limitation of SDS-PAGE technique, in Villemure *et al*. study, could not reveal the particular spots in several forms as shown by 2D-PAGE results obtained from this study. Thus this is the first study reported the 3 different pI/M_r spots of PREDICTED: seminal plasma protein PDC 109-like. The similar evidence was also reported in bovine seminal plasma by Thepparat (2012) and Thepparat *et al*. (2012).

PDC-109, in bovine, is referred to the identical of BSP-A1 and BSP-A2 in their primary structure of 109 amino acid residue and differed only in the degree of glycosylation (Manjunath and Sairam, 1987). It is the member of BSP protein family which is largely secreted from the seminal vesicles (Nauc and Manjunath, 2000). This protein binds to spermatozoa membrane at ejaculation (Manjunath *et al*., 1993). According to the report of Zaia (2004) the glycosylation proteins increases the complexity of protein molecules and causes them to migrate as diffuse spots on SDS-PAGE gels, it make room to

believe that the various pI/M_r values of PREDICTED: seminal plasma protein PDC 109-like which found in this study occurred from glycosylation.

The function of PDC-109 involved in sperm capacitation by anchoring heparin to the sperm surface and by stimulating sperm cholesterol efflux (Moreau *et al*., 1998). In addition, PDC-109 also plays a role in forming an oviductal spermatozoa reservoir by enabling sperm to bind to oviductal epithelium (Gwathmey *et al*., 2003). Furthermore, in vitro study showed the functions of PDC-109 as a molecular chaperone, suggesting that it may assist the proper folding of proteins involved in the bovine spermatozoa capacitation pathway (Sankhala and Swamy, 2010).

For PREDICTED: spermadhesin Z13-like, this protein also expressed in a large amount on 2D gel. It should be the same as spermadhesin Z13 which reported in bovine seminal plasma as its similar M_r (Moura *et al*., 2006). Rucker *et al*. (2010) also reported the bodhesin-2, belong to a new family of spermadhesins, is the interested major seminal plasma protein.

Spermadhesin is a seminal plasma protein made up of two disulfide-linked 13 kDa subunits. This protein is a non-glycosylated dimer that presents one interchain disulfide bond and does not show heparin-binding properties. The function of this protein is thought to play a role in fertilization (Tedeschi *et al*., 2000). Moura *et al*. (2007) also proposed that this protein potentially involved in sperm motility.

PREDICTED: caltrin-like may be the same as caltrin protein which has been reported in bull and rat as it is a small and basic protein (Agustin *et al*., 1987 and Dematteis *et al*., 2008). Both in bovine and rat, this protein binds to spermatozoa head at ejaculation prevent the occurrence of the spontaneous acrosomal exocytosis along the female reproductive tract. Consequently, more competent spermatozoa with intact and functional acrosome would be available in the oviduct to participate in fertilization (Agustin *et al*., 1987, Coronel *et al*., 1992, and Dematteis *et al*., 2008).

The results obtained from 2D-PAGE and SDS-PAGE revealed the inconsistence of PREDICTED: caltrin-like expression. It may be the cause of 1 spot with shown on 2D gel containing many proteins identity caltrin and other associated proteins. The further study, especially western blot performing, of this protein is very interesting because of its distinguishing among bucks. It may reveal the important role of this protein in spermatozoa functions.

Conclusion

The separation of caprine seminal plasma proteins by using 2D-PAGE and LC MS/MS techniques revealed the expression of major seminal plasma proteins at molecular weight approximately 10-16 kDa which were PREDICTED: seminal plasma protein PDC 109-like, PREDICTED: spermadhesin Z13-like, and PREDICTED: caltrin-like. The 4 expression patterns of PREDICTED: seminal plasma protein PDC 109-like were different among the 20 experimental bucks, however, did not associated with buck fertility. The PREDICTED: caltrin-like clearly showed presence or absence on 2D gels of each buck. However, this study could not clarify the expression of this protein although SDS-PAGE and LC MS/MS were used to carry out.

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Appendix

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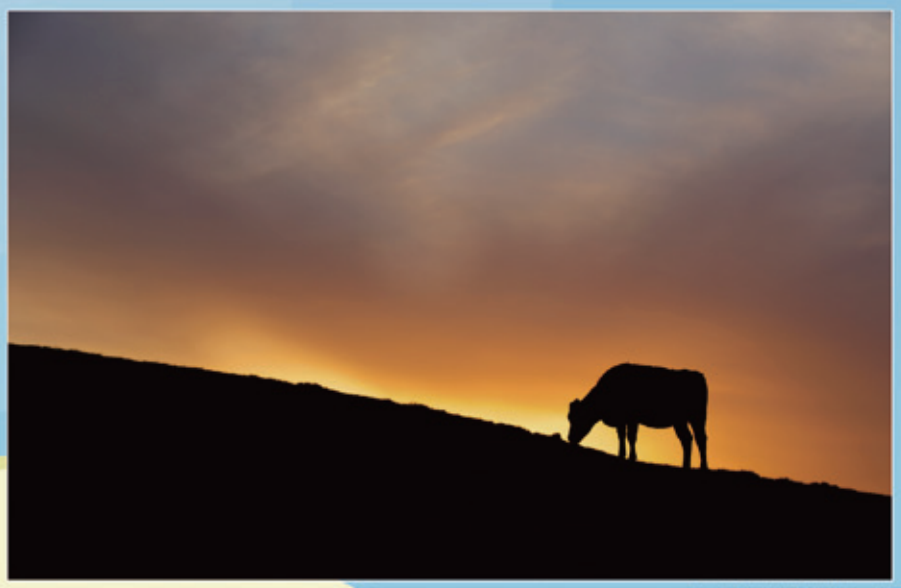

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Major proteins in caprine seminal plasma

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Major proteins in caprine seminal plasma

Introduction

Seminal plasma is the fluid mainly secreted from accessory sex glands. The important of seminal plasma is that it contains the potential proteins which affecting male fertility. Some of seminal plasma proteins bind to ejaculated spermatozoa membrane and make spermatozoa to acquire the fertilization capacity with female gamete. For instance, in bovine, the heparin binding proteins (HBPs) are the other name of bovine seminal plasma proteins (BSP proteins) and largely secreted from the seminal vesicles (Manjunath et al., 1994). These proteins bind to spermatozoa membrane at ejaculation (Manjunath et al., 1994) and participate in the process of fertilization (Therien et al., 1995).

A few studies of seminal plasma proteins of goat have been reported. The isolation and characterization of gelatin-binding proteins from goat seminal plasma indicated that goat seminal plasma proteins (GSP proteins) homolog to BSP proteins which exist in several forms in each species and possibility play a common biological role (Villemure et al., 2003). In addition, Heparin-affinity proteins (HAPs) are under seasonal control and associated with sperm function during breeding and non-breeding seasons (Falci et al., 2002). The bodhesin-2, belong to a new family of spermadhesins, is the interested major seminal plasma protein in goat (Rucker et al., 2010).

Recently, proteomic approaches such 2D-PAGE and MS have been introduced to unveil the molecular basis of several physiological events and provide the information understanding the framework of biology. The underlying mechanism of fertility involved particularly with seminal plasma proteins would be a straightforward approach to improve buck fertility.

Objective

The objective of this study was to investigate the proteins in caprine seminal plasma using 2D-PAGE and MS techniques.

Methodology

Seminal plasma collection

Semen samples were collected from 5 Boer bucks with between 2 to 5 years of age. The first ejaculated semen was used in this study. Seminal plasma was obtained from the semen by centrifuging at 800×g for 5 min. The supernatant seminal plasma was then transferred to 1.5 ml tubes and centrifuged at 10,000×g for 60 min at 4°C. The supernatant of each sample was divided into 1.5 ml aliquots and stored at -70°C for further measuring protein concentration and performing the 2D-PAGE.

Two-dimensional polyacrylamide gel electrophoresis

Seminal plasma samples were assayed for protein concentration (Bradford, 1976) using bovine serum albumin as the standard before performing the 2D-PAGE. The 2D-PAGE was performed according to O'Farrell (1975). Briefly, the isoelectric focusing (IEF) was performed in Isoelectric Focusing System in 13 cm IPG dry strips with a pH range 3-10. Prior to the IEF, the seminal plasma sample was diluted to 200 µg of protein in 250 ml of re-hydration solution (7M urea, 2M thiourea, 4% w/v CHAPS, 2% w/v DTT, 0.5% v/v IPG buffer, 0.002% Bromophenol blue). This mixing solution was loaded to the Immobiline DryStrip Reswelling tray, the IPG dry strip was placed with the gel side down and overlayed with Immobiline DryStrip cover fluid. The IPG dry strip was allowed to re-hydrated overnight (10-20 h).

The focusing was performed on an Isoelectric Focusing Unit using the following 4 steps: (i) step and hold 500 V for 500 Vh (ii) gradient up to 1000 V for 800 Vh (iii) gradient up to 8000 V for 11300 Vh and (iv) step and hold 8000 V for 7400 Vh. The focused was equilibrated in a buffer (6 M 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 Iodoacetate (IAA) for 30 min.
After being equilibrated, the strip was done in a second dimension of 12.5% SDS-polyacrylamide gel. The low molecular weight standard, range 14-97 kDa, was loaded to the gel. The equilibrated IPG gel strip was embedded in a sealing solution (0.5% agarose in 25 mM Tris-base, 192 mM glysine, 0.1% SDS and 0.002% bromophenol blue).
The vertical setup was used using 25 mA/gel. After that the gel was stained in colloidal Coomassie Brilliant Blue G-250 (0.08% Coomassie Brilliant Blue G-250, 8% ammonium sulfate, 0.8% phosphoric acid, 20% methanol) overnight. The gel was de-stained with de-ionized water for at least 24 h or until the background was clear. At least one duplicate gel was performed for each sample. Each gel was scanned with an ImageScanner System and analyzed spots by ImageMasterTM 2D Platinum software.
LC MS/MS
The major expression protein spots, with a large amount of relative protein content were cut from 2D-gel and identified using the LC MS/MS technique. Each spot sample was digested with trypsin enzyme then the digested peptides were analyzed by LC/MS/MS mass spectrometry, (Thermo Electron). The LC separation and MS analysis details were: HPLC system, Finnigan SurveyorTM MS pump with a flow splitter Column, 0.18 × 100 mm C18 (Thermo Electron) particle size 5µm Flow rate, 100µl/min Mobile phase A, water with 0.1% formic acid Mobile phase B, acetonitrile with 0.1% formic acid Gradients, 7-60 %B in 30 min 65-80 %B in 5min and hold 5min, 80-7 %B in 2min Mass Spectrometer, Finnigan LTQ Ionization mode, NanoSpray, positive ion Capillary temperature, 200 C Spray needle voltage, 1.8 KV Mass range, 400-1,600 m/z Scan sequence, Full-scan MS, MS/MS scan Acquisition modes, Normal, Data DependentTM and Dynamic ExclusionTM. The molecular weight values of the trypsinized peptides obtained by LC MS/MS were then used to identify the predicted proteins using MASCOT web-bases search engines.

Results and discussions

After performing the protein separating using 2D-PAGE and stained with colloidal Coomassie Brilliant Blue, more than 213 protein spots with a pI of pH 3-10, and M_r of 10-97 kDa could be detected in seminal plasma of an experimental buck (Figure 1). Of all these spots, there were proteins at molecular weight approximately 14-17 kDa expressed in a large amount on the gel. Identification using MS technique and MASCOT web-bases search engines found that the major seminal plasma proteins matched to PREDICTED: seminal plasma protein PDC 109-like (NCBI accession no. was gi|548504897) and PREDICTED: Spermadhesin Z13-like (NCBI accession no. was gi|548523829), in *Capra hircus*. The predicted means derived from a genomic sequence.
The major seminal plasma proteins of the buck which found in this study were PDC 109 and Spermadhesin Z13. These proteins expressed in a large amount on the 2D gel. For PDC 109, it expressed in 5 different spots of pI and MW. This, however, is the first study to report the presence of PDC 109 in different expression. The expression profile of PDC 109 of the buck was different to the bull (Thepparat et al., 2012).
PDC-109 is the BSP A-1/-A2, belongs to the BSPs proteins family, which has been reported in bovine. The function of PDC-109 involved in the fertilization process. In vitro study showed the functions of PDC-109 as a molecular chaperone, suggesting that it may assist the proper folding of proteins involved in the bovine spermatozoa capacitation pathway (Sankhala and Swamy, 2010). In addition, PDC-109 also plays a role in forming an oviductal spermatozoa reservoir by enabling sperm to bind to oviductal epithelium (Gwathmey et al., 2003 Ignotz et al., 2001).
Spermadhesin Z13 is a seminal plasma protein made up of two disulfide-linked 13 kDa subunits. The function of this protein is thought to play a role in fertilization (Tedeschi et al., 2000). Moura et al. (2007) also proposed that this protein potentially involved in sperm motility, but the mechanism for an inverse relationship with fertility is unclear.

Conclusions

It can conclude from this study that PDC 109 and Spermadhesin Z13 were the major proteins in seminal plasma of the buck. The expression of PDC 109 showed 5 different spots of pI and MW.

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KEYWORD : Caprine seminal plasma proteins, 2D-PAGE

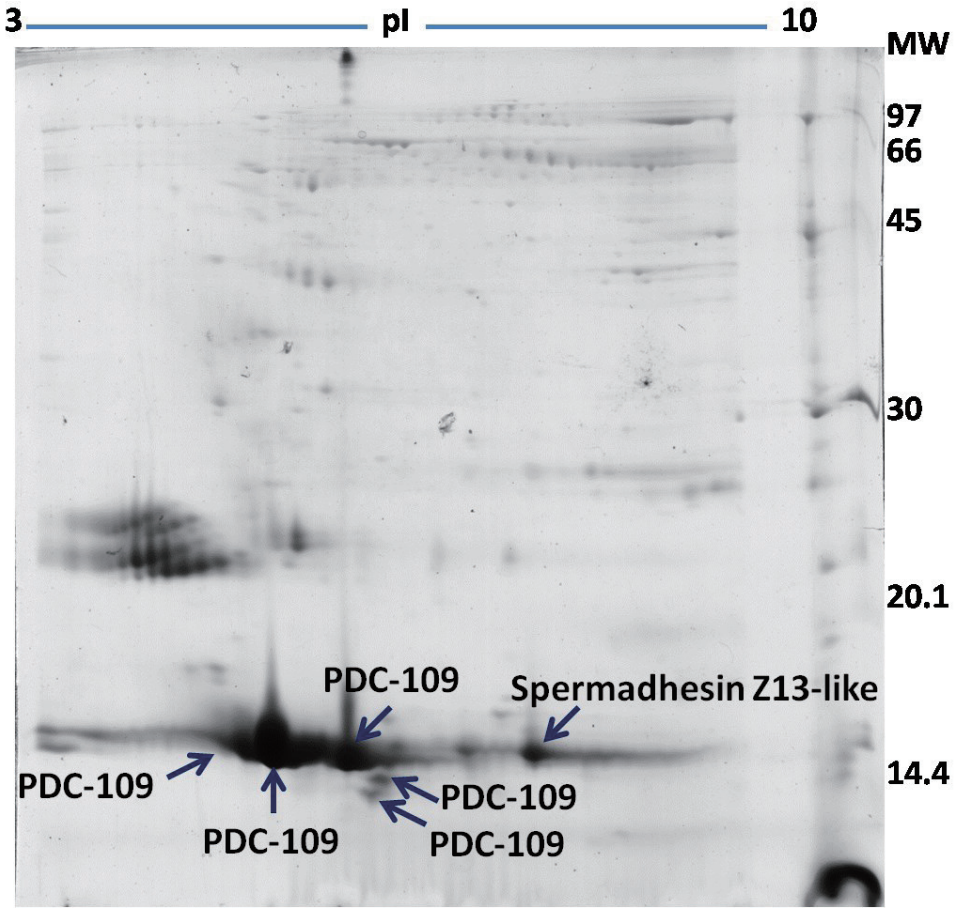

Figure 1 2D-gel of caprine seminal plasma protein. 200 μg of protein was electro-focused on 13 cm DryStrip gel (range, pH 3-10). SDS-PAGE was conducted on a 12.5% acrylamide gel plate. The M_r standard used ranged between 14.4-97 kDa. In this figure, M_r is on the Y-axis and pI (3-10) on the X-axis. Colloidal Comassie Brilliant Blue G-250 was used for the protein staining.

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