

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(8): 1139-1143
www.biochemjournal.com
Received: 08-06-2024
Accepted: 14-07-2024

Dhanu Kumar Murasing
G.B. Pant University of
Agriculture and Technology,
Pantnagar, Uttarakhand,
India

Sofi Imran Ul Umar
(1) ICAR-Indian Institute of
Agricultural Biotechnology,
Ranchi, Jharkhand India
(2) Birsa Agricultural
University, Ranchi, India

Soumen Naskar
ICAR-Indian Institute of
Agricultural Biotechnology,
Ranchi, Jharkhand India

Mridula Sharma
G.B. Pant University of
Agriculture and Technology,
Pantnagar, Uttarakhand,
India

Corresponding Author:
Mridula Sharma
G.B. Pant University of
Agriculture and Technology,
Pantnagar, Uttarakhand,
India

Isolation of plasma membrane proteins from bovine spermatozoa using ultracentrifugation

Dhanu Kumar Murasing, Sofi Imran Ul Umar, Soumen Naskar and Mridula Sharma

DOI: <https://doi.org/10.33545/26174693.2024.v8.i8So.2003>

Abstract

In this study, we employed 10% SDS-PAGE to analyze total and plasma membrane proteins from bovine spermatozoa, revealing an average of 17 bands for total sperm proteins and 12 bands for plasma membrane proteins. The molecular weights of the proteins ranged from 21 to 250 kDa in cell lysates and 30 to 250 kDa in plasma membrane proteins. Prominent bands at approximately 26, 50, 60, 67, 100, and 120 kDa were observed in total sperm proteins, while bands at approximately 40, 80, 95, and 110 kDa were noted in plasma membrane proteins. These results highlighting both quantitative and qualitative differences in low molecular mass proteins. The higher molecular weight bands in total sperm proteins may be attributed to variations in protein concentration. Further, 2D electrophoresis identified over 60 protein spots in the sperm membrane, with molecular weights ranging from 37 to 200 kDa. These findings underscore the complexity and variability of sperm protein profiles and suggest that they could improve fertility assessments and semen preservation methods. Future research, including LC-MS analysis and immunoblot techniques, is needed for detailed protein identification and to advance our understanding and application of reproductive technologies.

Keywords: Plasma membrane proteins, bovine, spermatozoa, SDS-PAGE, 2D electrophoresis

Introduction

In the 1980s, breakthroughs in molecular biology, including gene cloning, sequencing, and expression analysis, set the stage for advancements in proteomics. Today, proteomic methods are widely employed for protein profiling, comparing protein expression across different organs and tissues, detecting and characterizing post-translational modifications, and investigating protein-protein interactions. Recent improvements in fractionation and labeling techniques have enhanced the ability to identify even the least abundant proteins. Recent proteomic studies have primarily focused on enhancing the resolution of protein separation and the comprehensive identification of proteins using gel electrophoresis and mass spectrometry. Wilkins *et al.*, (1996) ^[15]. However, the separation of complex mixtures of proteins and peptides remains one of the most challenging aspects of proteomics. du Plessis *et al.*, (2011) ^[2].

Fertilization of an ovum by a spermatozoon involves a series of membrane-mediated events, including the acrosome reaction, release of acrosomal contents, and penetration through the zona pellucida of the ovum, which allows for contact and fusion with the vitelline membrane. Agrawal *et al.*, (1988) ^[1]. Given the significant changes that occur in the sperm plasma membrane during epididymal maturation, capacitation, the acrosome reaction, and fertilization, analyzing specific domains of the sperm membrane at the biochemical level is crucial for understanding sperm maturation and fertilization processes. Noland *et al.*, (1983) ^[9]. It is now well-established that mammalian sperm have specific plasma membrane components involved in binding to the zona pellucida. (Peterson and Hunt, 1989) ^[11]. Early studies, often using porcine or bovine spermatozoa, employed methods such as sonication, Lunstra *et al.*, (1974) ^[5]. Detergent extraction, (Multam-ki and Pelliniemi, 1973) ^[8], or mechanical homogenization. (Zahler and Doak, 1975) ^[17]. However, these methods have limitations: 1) they require large numbers of spermatozoa, making them impractical for isolating membrane proteins; 2) they may inhibit enzyme activity, denature membrane proteins, or disrupt membrane integrity; and/or 3) they can result in plasma membrane

preparations contaminated with materials from other sperm structures. (Peterson and Hunt, 1989) [11]. Currently, plasma membrane-enriched fractions from spermatozoa are purified using discontinuous sucrose density gradient centrifugation. Noland *et al.*, (1983) [9]. Despite this, the biochemical properties of the mammalian sperm plasma membrane are not yet fully understood. Given the critical role the plasma membrane plays in various fertilization events, a thorough understanding of sperm membrane properties and changes is essential. Knowledge of the structure of the plasma membrane overlying the acrosome is crucial for detailing the molecular aspects of these processes. Mack *et al.*, (1986) [6].

Recent proteomic techniques, including 1D polyacrylamide gel electrophoresis (1D-PAGE), 2D polyacrylamide gel electrophoresis (2D-PAGE), mass spectrometry (MS), and differential in gel electrophoresis (DIGE), have enabled the identification of numerous sperm-specific proteins. These approaches have enhanced our understanding of the protein functions involved in sperm processes such as motility, capacitation, the acrosome reaction, and fertilization. du Plessis *et al.*, (2011) [12]. This study aims to describe methods for isolating and characterizing total and plasma membrane proteins from bovine spermatozoa. Such methods will support future advancements in bioinformatics and contribute to the understanding of growth, development, and reproduction, potentially serving as biomarkers for detecting diseases or sperm impairments.

Materials and Methods

Preparation of sample

For this study Frozen Sahiwal Bull semen was used. The semen sample containing 4×10^6 spermatozoa was thawed at 37°C for 30 seconds and centrifuged at 800 x g at 25°C for 20 min to remove the diluents. The pellets were resuspended with 1 mL PBS, pH 7.4 (Bio-Rad, USA) and washed twice at 800 x g for 10 min. to remove the excess diluents.

Isolation of total sperm protein

The total sperm protein from bull sperm was isolated with slight modification of Yamamoto *et al.*, (2016) [16]. Briefly, the sperm pellets were diluted with 200 μ L of buffer A containing 0.1% (v/v) NP-40 (Sigma, France, Cat. No. 74385), 50 mM Tris-HCl, pH 8.0 (Himedia, India, Cat. No. MB029), 0.5 mM DTT (Sigma, Cat. No. 9779), 2% (v/v) protease inhibitor cocktail (Sigma, P8340) and 1% (v/v) phosphatase inhibitor cocktail (Sigma, Cat. No. P0001). After that the sample was homogenized with pestle homogenizer for 20 times and 22G needle for 2-3 times followed by 100 μ L of 2x RIPA lysis buffer containing 20 mM Tris-HCl, pH 8.0, 300mM NaCl (Himedia, Cat. No. MB023), 2% (v/v) NP-40, 1% (w/v) sodium deoxycholate (Sigma, Cat. No. D6750), 0.2% (w/v) sodium dodecyl sulfate (Sigma, Cat. No. L3771), 2% (v/v) protease inhibitor cocktail, 1% (v/v) phosphatase inhibitor cocktail was added and incubated for 1 h at 4°C. The centrifuged at 18,000 x g at 4°C for 25 min. the supernatant were collected in a clean tube. The quantification of protein-enriched fraction was assessed by Micro BCA Protein Assay Kit (Thermo Scientific, USA, Cat. No. 23235) as per manufacturer's guidelines.

Isolation of a sperm plasma membrane protein

Plasma membrane was isolated from sperm cells following the method of Smart *et al.*, (1995) [13] with modifications. After thawing the sperm samples were washed thrice with

PBS for 10 min each and the pellet was harvested in 3 mL of buffer A (250 mM sucrose, 20 mM tricine, 1 mM EDTA, pH 7.8). Then we performed centrifugation with the following parameters: 1,000 x g for 5 minutes at 4 °C. After this cell pellet was taken in 1 mL of buffer A and hand homogenized with 20 strokes. The homogenate was transferred to a 1.5 mL tube and centrifugation was performed at 1,000 x g for 10 minutes at 4 °C. The post nuclear supernatant (PNS) was collected and stored on ice. Using the same pellet, Steps of homogenation were repeated. From both PNS supernatants were pooled and kept on ice. The PNS was layered on the top of 5 mL of 30% Percoll in buffer A in a 7 mL thick-walled tube. Performed centrifugation at 84,000 x g for 30 minutes at 4 °C using Ultracentrifuge machine (himac, S150FNX). Collected the PM fraction and store in 3 mL microtubes. Percoll was removed by centrifuging at 105,000 x g for 90 minutes at 4 °C using the Ultracentrifuge. Carefully removed the PM and placed in a new high performance microtube. If necessary, repeat the centrifugation process. The quantification of protein-enriched fraction was assessed by Micro BCA Protein Assay Kit (Thermo Scientific, USA, Cat. No. 23235) as per manufacturer's guidelines.

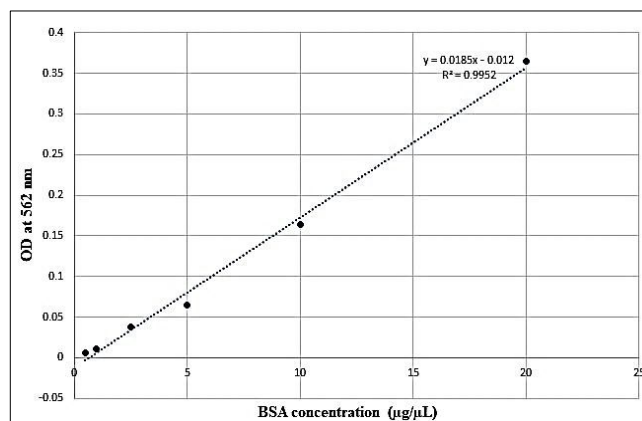


Fig 1: The standard BCA estimations curve for estimation of total sperm protein

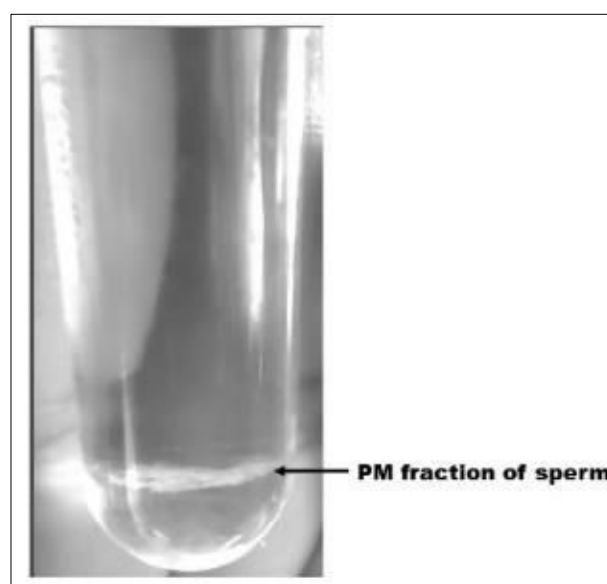


Fig 2: Plasma membrane fraction of sperm

Protein Characterization by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as per the method of Laemmli. (1970) [3]. Briefly, two separate gels were prepared: a 10.0% separating gel and a 5.0% stacking gel, each using the standard formulation detailed in Table 1. Samples consisting of sperm protein were separately prepared by mixing respective samples with sufficient (4X) sample buffer [200 mM Tris (pH 6.8), 8% SDS, 0.4% Bromophenol blue, 40% Glycerol, 400 mM β -mercaptoethanol] and denatured the protein sample by heating at 95 °C for 5 minutes. Wells were charged with 25 μ L of respective heat-treated samples. One well was charged with 5 μ L pre-stained standard

protein ladder of molecular weight ranging from 250 to 10 kDa (Biorad, Cat. No. 161-0374). Stained with Coomassie Brilliant Blue R-250 (MP Biomedicals, Cat. No. 190682) for overnight in an orbital shaker followed by destained. Protein bands were visualized in Gel documentation system [Gel Doc XR+ (Bio Rad, USA)] for detection of number of proteins with their molecular weights. The molecular weights of sperm protein fraction were determined by molecular weight analysis tool of Gel documentation system (Image LabTM software-Bio-Rad, USA) by comparing with standard molecular weight marker.

Table 1: Composition of gel for protein profiling of the sperm by SDS-PAGE

Reagents	Separating gel (12%)	Stacking gel (5%)
Double distilled water	3.3 mL	3.4 mL
Acrylamide/Bis Acrylamide (30%/0.8% w/v)	4.0 mL	0.83 mL
1.5 M Tris-HCl, pH 8.8	2.5 mL	-
0.5 M Tris-HCl, pH 6.8	-	0.63 mL
Sodium dodecyl sulphate (SDS) (10% w/v)	0.1 mL	0.05 mL
Ammonium persulfate (APS) (10% w/v)	0.1 mL	0.05 mL
TEMED	10 μ L	5 μ L

Two-Dimensional Electrophoresis (2D-PAGE)

Two-dimensional electrophoresis of sperm proteins was performed according to the method of O'farrells. (1975) [10] with slight modifications. The first dimension utilizes isoelectric focusing (IEF) to separate proteins based on their isoelectric points. In the second dimension, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is employed to separate proteins according to their molecular size. The experiment utilized the ReadyPrep 2-D starter kit (Bio-Rad, Cat. No. 163-2105). First, the desired number of pH 4-7 ReadyStrip IPG strips were removed from the -20 °C freezer and set aside. The rehydration buffer/sample buffer was prepared by reconstituting the lyophilized sperm sample to a final volume of 125 μ L, containing approximately 169 μ g of protein. The 7 cm pH 4-7 IPG strips were removed from their backing and carefully positioned, gel side down, onto the sample. The rehydration tray was left at room temperature for 11-16 hours to allow the IPG strips to rehydrate and the protein sample to be loaded. To ensure adequate hydration, 8 μ L of nanopure water was added to each paper wick. The focusing tray was then placed into the PROTEAN IEF cell (Bio-Rad, USA) at 20 °C with a maximum current setting of 50 μ A per strip, following this program: 50 V/h with a linear ramp (0-300 V), 50 V/h with a linear ramp (300-600 V), 500 V/h with a linear ramp (600-2000 V), 1000 V/h with a linear ramp (2000-4000 V), and 2000 V/h with a rapid ramp (4000-50,000 V) up to a total of 52 kVh. After IEF, the IPG strips were equilibrated in equilibration solution I for 15 minutes, followed by equilibration in solution II containing 5% iodoacetamide instead of DTT. The IPG strips were positioned on the back plate of the SDS-PAGE gel, and an agarose solution was poured into the IPG well of the gel. The agarose was left to

solidify for 5 minutes before proceeding. The 2D-PAGE gel was then stained with Coomassie Brilliant Blue R-250 for 1 hour on an orbital shaker. The stained gel was destained and visualized using a Gel Doc XR+ system (Bio-Rad, USA) to preserve the gel image.

Results and Discussion

SDS-PAGE analysis of sperm proteome

Total proteins and plasma membrane proteins were resolved using 10% SDS-PAGE, as shown in Figure 3. The gels of cell lysates revealed an average of 17 bands for total sperm proteins and 12 bands for plasma membrane proteins (Figure 3). The molecular weights of the proteins in the cell lysate ranged from 21 to 250 kDa, while the plasma membrane proteins ranged from 30 to 250 kDa. Prominent bands at approximately 26, 50, 60, 67, 100, and 120 kDa were identified in the total cell lysate, whereas bands at approximately 40, 80, 95, and 110 kDa were observed in the plasma membrane proteins. These results align with the findings of Marques *et al.*, (2000) [7], who noted both quantitative and qualitative differences in low molecular mass proteins in bovine spermatozoa. The higher molecular weight bands observed in total sperm proteins compared to plasma membrane proteins may be attributed to differences in protein concentration. Similar findings were reported by Sharma *et al.*, (2022) [12], and Laxmivandana *et al.*, (2021) [4]. Identifying sperm plasma membrane proteins involved in the fertilization process could contribute to the development of cost-effective biochemical tests for assessing semen fertility in commercial artificial insemination (AI) operations. This identification could also improve protocols for semen cryopreservation.

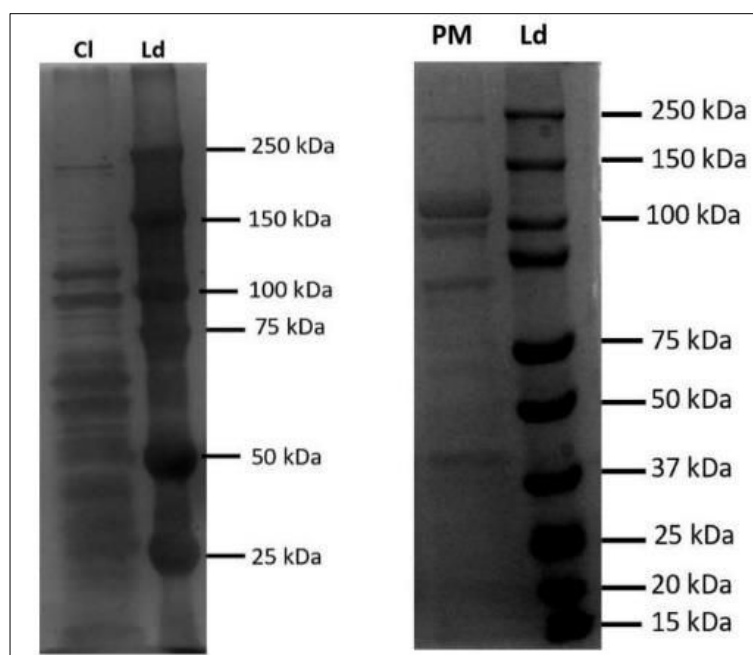


Fig 3: SDS-PAGE of total cell lysate and plasma membrane from bovine sperm (Cl- Cell lysate, PM- Plasma membrane and Ld- Protein ladder)

Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) of bovine sperm

To investigate the molecular characterization of sperm proteins, we analyzed the protein profile of the sperm membrane using 2D electrophoresis. More than 60 protein spots were detected, with molecular weights ranging from 37 to 200 kDa (Figure 4). These results are consistent with the findings of Van Tilburg *et al.*, (2013) ^[14], who identified 68 spots. A greater number of protein spots may be obtained by using a larger number of sperm or fresh ejaculates. Further studies are needed to identify the separated proteins using LC-MS analysis followed by immunoblot techniques.

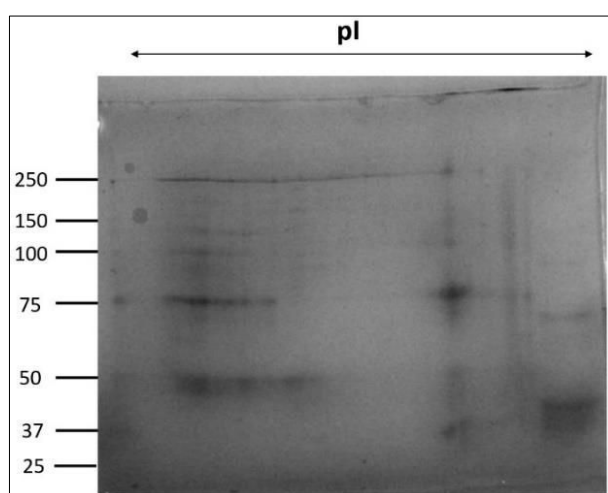


Fig 4: Two-dimensional separation of sperm proteins. 2-D was performed using a pH range of 4-7 in the first dimension and SDS-PAGE (12.5 %) in second. The protein loading was 169 µg, and the gel was stained using a Coomassie Brilliant Blue R-250 procedure

Conclusion

We identified a diverse range of sperm-associated protein bands using 1D SDS-PAGE and protein spots through 2D SDS-PAGE in bovine spermatozoa. This study highlights the complexity and variability of sperm protein profiles and

suggests that these findings could enhance fertility assessments and semen preservation methods. Further detailed analysis and identification of these proteins will be essential for advancing our understanding and application of reproductive technologies.

Acknowledgment

The present study is jointly planned by GBPUAT, Pantnagar and ICAR-IIAB, Ranchi, and carried out at ICAR-IIAB. The funding support is received from the Science and Engineering Research Board (Govt. of India) through research projects 2019/000437 and 2023/000377 (to SN) and ICAR-IIAB. We also thank the Vice-Chancellor, GBPUAT and the Director, ICAR-IIAB for facilitating collaboration in research and academics through MoU.

References

1. Agrawal P, Magargee SF, Hammerstedt RH. Isolation and characterization of the plasma membrane of rat cauda epididymal spermatozoa. *J Androl.* 1988;9(3):178-189
2. du Plessis SS, Kashou AH, Benjamin DJ, Yadav SP, Agarwal A. Proteomics: A subcellular look at spermatozoa. *R B E; c2011*;9.
3. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nat.* 1970;227(5259):680-685.
4. Laxmivandana R, Patole C, Sharma TR, Sharma KK, Naskar S. Differential proteins associated with plasma membrane in X- and/or Y-chromosome bearing spermatozoa in indicus cattle. *Reprod Domest Anim.* 2021;56(6):928-935.
5. Lunstra DD, Clegg ED, Morre DJ. Isolation of Plasma Membrane from Porcine Spermatozoa. *Prep Biochem.* 1974;4(4):341-352.
6. Mack SR, Everingham J, Zaneveld LJ D. Isolation and partial characterization of the plasma membrane from human spermatozoa. *J Exp Zool.* 1986;240(1):127-136.
7. Marques VA, Goulart LR, Silva AE D. Variations of protein profiles and calcium and phospholipase A2

- concentrations in thawed bovine semen and their relation to acrosome reaction. *Genet Mol Biol.* 2000;23:825-829.
8. Multamäki S, Pelliniemi LJ. Ultrastructure of Subcellular Fractions of Bull Spermatozoa after Hyamine Treatment. *Z Zellforsch.* 1973;144.
 9. Noland TD, Olson GE, Garbers DL. Purification and Partial Characterization of Plasma Membranes from Bovine Spermatozoa. *Biol Reprod.* 1983;29.
 10. O'Farrells PH. High Resolution Two-Dimensional Electrophoresis of Proteins*. *Biol Chem.* 1975;25(10).
 11. Peterson RN, Hunt WP. Identification, isolation, and properties of a plasma membrane protein involved in the adhesion of boar sperm to the porcine zona pellucida. *Gamete Res.* 1989;23(1):103-118.
 12. Sharma V, Verma AK, Sharma P, Pandey D, Sharma M. Differential proteomic profile of X- and Y- sorted Sahiwal bull semen. *Vet Sci Res.* 2022;144:181-189.
 13. Smart EJ, Ying YS, Mineo C, Anderson RGW. A detergent-free method for purifying caveolae membrane from tissue culture cells. *Cell Biol.* 1995;92.
 14. Van Tilburg MF, Rodrigues MAM, Moreira RA, Moreno FB, Monteiro-Moreira ACO, Cândido MJD, Moura AA. Membrane-associated proteins of ejaculated sperm from Morada Nova rams. *Theriogenology.* 2013;79(9):1247-1261.
 15. Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez JC, Hochstrasser DF. From proteomes to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnol.* 1996;14(1):61-65.
 16. Yamamoto N, Yamashita Y, Yoshioka Y, Nishiumi S, Ashida H. Rapid preparation of a plasma membrane fraction: Western blot detection of translocated glucose transporter 4 from plasma membrane of muscle and adipose cells and tissues. *Curr Protoc Protein Sci.* 2016; 29.18.1-29.18.12.
 17. Zahler WL, Doak GA. Isolation of the outer acrosomal membrane from sperm bull. *Biochim Biophys Acta.* 1975;406.