PURIFICATION AND QUANTIFICATION OF FIBRONECTIN TYPE II PROTEINS IN MALABARI BUCK SEMINAL PLASMA

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Abstract

The study was conducted to purify and quantify Fn2 type (Fibronectin type II) proteins of Malabari buck seminal plasma. Quantification of whole seminal plasma protein did not reveal any significant difference between bucks with good and poor semen freezability, while significant difference was observed in the quantity of Fn2 proteins in the two groups of bucks. SDS-PAGE analysis revealed four gelatin binding proteins with apparent molecular weight 14kDa, 15kDa, 20kDa and 22kDa.

Keywords: *Fn2 type proteins, Buck seminal plasma, Affinity chromatography*

Fibronectin type – II proteins (Fn2 type proteins) constitute the major class of seminal plasma proteins. In bovines, the Fn2 type seminal plasma proteins are termed as Binder of sperm proteins (BSP). Manjunath *et al*. (1987) reported that the BSPs in bovines consisted of three major proteins namely BSP-A_1/ A_2, BSP-A_3 and BSP- 30 kDa. BSP-A_1 and BSP-A_2were called collectively as PDC-109 (Calvete *et al.,* 1996). In goats, Villemure *et al.*(2003) reported four proteins, namely, GSP-14kDa,GSP-15kDa, GSP-20kDa and GSP-22kDa as BSP-homologous proteins.

BSPs have got two tandem fibronectin gelatin binding domains. These domains interact with collagen and gelatin. The proteins have been reported to play a major role in fertilization, including sperm reservoir formation and sperm cryodamage. The treatment of buffalo cauda epididymal spermatozoa with Fn2 proteins was found to result in a highly significant and dose dependent loss of post thaw viability when compared to untreated spermatozoa (Harshan *et al.,* 2006). Villemure *et al.* (2003) carried out gelatin affinity chromatography to isolate Fn2 proteins of buck seminal plasma with molecular weights of GSP-14 kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa. Eight molar urea was used for elution of BSP proteins of Vechur bull seminal plasma (Noolvi, 2015). Shiny (2011) recorded a mean total protein concentration of 32.38 ± 1.40 mg/ml in seminal plasma of Malabari bucks, while John (2016) recorded a mean protein concentration of 75.82 ± 7.95

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and 67.07 ± 10.90 mg/ml in the seminal plasma of Malabari bucks with good and poor semen freezability, respectively. Purification and quantification of Fn2 type proteins in Malabari buck seminal plasma have not been reported. The present study was designed to isolate and quantify the Fn2 type proteins from Malabari buck seminal plasma.

Materials and Methods

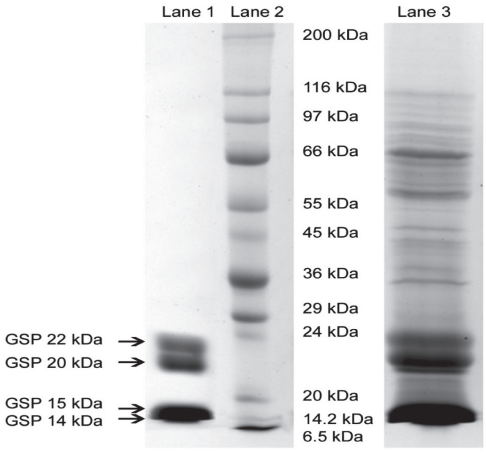
Six adult apparently healthy Malabari bucks, of which three were having a post-thaw sperm motility of greater than 35% (good semen freezability) and three having a post-thaw sperm motility of less than 25% (poor semen freezability) were selected for the study. Six ejaculates from each of the six bucks under study were collected using artificial vagina and immediately supplemented with anti-protease cocktail (P 8340, Sigma Aldrich, USA) at the rate of 10 µl per ml of semen. The ejaculates were centrifuged at 1500 × g for 15 min at room temperature and the supernatant clarified immediately at a subsequent centrifugation at 10000 × g for 30 min at 5 °C to harvest seminal plasma. The harvested seminal plasma was stored below -20°C until analysis.

For isolation of Fn2 type proteins, gealtin affinity chromatography was carried out as per Manjunath *et al.* (1987). Thawed seminal plasma from six ejaculates of each of the six bucks were pooled separately and subjected to gelatin affinity chromatography. The affinity chromatography was carried out in a gelatin column prepared as per Manjunath *et al.* (1987). The column was equilibrated with 30 ml of equilibration buffer. One ml of clear pooled seminal plasma was extended three times with the equilibration buffer (50 mM Tris – HCl, 0.15 M NaCl, 5 mM EDTA, 2 mM PMSF and 0.025 per cent sodium azide) and loaded onto the equilibrated column. Flow was stopped for about fifteen minutes to allow the proteins to interact with the column matrix. Washing of the column was performed with twenty bed volume of equilibration buffer to remove un-adsorbed proteins and the wash was kept separately. The adsorbed gelatin binding proteins were eluted in three ml fractions with twenty bed volumes of elution buffer (50 mM Tris-HCL, 10 mM EDTA, 2 mM PMSF, 8 M urea and 0.025 per cent sodium azide). The eluted fractions were monitored by absorbance at 280 nm and an absorbance of near zero was considered as indicative of complete elution. The wash which was kept initially, were again passed through the activated gelatin column to harvest any leftover gelatin binding proteins present in the wash and the bound proteins were eluted with elution buffer. The column, after washing in equilibration buffer, was stored in storage buffer (0.5 M NaCl with 0.05 per cent sodium azide) at 5°C. The whole seminal plasma protein content and gelatin binding protein fraction were quantified as per Lowry *et al.* (1951).

The protein profile of whole seminal plasma and gelatin binding fraction were performed by Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE, 10 per cent gel) as per Laemmli (1970) in a Mini-PROTEAN tetra electrophoresis system (Bio-Rad, USA). The gel was stained using Coomassie brilliant blue R 250 staining for two hours. Gel imaging was done in Chemi Doc™ MP Imaging System (Bio-Rad, USA).

Results and Discussion

The mean total protein content of whole seminal plasma of Malabari bucks with good and poor freezability was found to be 61.38 ± 6.13 and 55.64 ± 3.1 mg/ml, respectively. The values did not differ significantly. The mean gelatin binding protein concentration of bucks with good and poor semen freezability was found to be 25.49 ± 2.09 mg/ml and 19.64 ± 0.64 mg/ml, respectively. Though the overall mean gelatin binding protein content of seminal plasma differed significantly ($p < 0.01$) between bucks with good or poor semen freezability, the overall proportion of gelatin binding protein with respect to total seminal plasma protein content did not differ significantly. The quantity and quality of seminal plasma proteins vary with animals and environmental factors such as season, temperature, nutrition and stress (Perez-Pe *et al.,* 2001). Buck seminal plasma proteins are influenced by season and are associated with spermatozoa function during breeding and non breeding seasons (La Falci *et al*., 2002). SDS – PAGE profiling of both whole



Lane 1:Gelatin binding proteins, Lane 2: Marker,
Lane 3: Whole seminal plasma proteins

Fig.1 SDS-PAGE profile of whole seminal plasma and gelatin-agarose binding fraction of Malabari buck seminal plasma proteins.

seminal plasma protein and gelatin binding protein fraction were done. The gelatin binding fraction denotes the GSP proteins of the buck seminal plasma as described by Villemure *et al.* (2003). It could be appreciated from the SDS-PAGE that GSP proteins form the major proteins of Malabari buck seminal plasma. Four gelatin binding protein bands with an apparent molecular weight of 14 kDa, 15 kDa, 20 kDa and 22 kDa could be identified (Fig.1).

In this study, isolation of Malabari buck seminal plasma Fn2 type proteins that are homologous to the BSP proteins were carried out. Gelatin-agarose affinity chromatography was done and four proteins with apparent molecular weight GSP-14kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa were isolated and characterized of by SDS – PAGE analysis. The result of this study is in concurrence with the observations made by Villemure *et al.*(2003).

The binding property of BSP proteins with type II domains to gelatin was used earlier by Manjunath *et al*. (1987) to purify the Fn2 proteins.

The Fn2 proteins have been reported to bind to spermatozoa membrane by their interaction with phospholipids like phosphatidylcholine and sphingomyelin. They also bind to high-density lipoproteins and mediate sperm binding to heparin like glycosaminoglycans found in oviductal and follicular fluids, thus playing an important role in fertilization (Desnoyers and Manjunath, 1992). The binding of protein to spermatozoa results in cholesterol efflux which in turn decreases the ratio of cholesterol/phospholipid, promoting spermatozoa capacitation (Therien *et al*., 1998). Cholesterol has been reported to have a stabilizing effect on cell membrane of sperms and its efflux provokes destabilization of the sperm membrane and increased permeability to certain ions like Ca^{2+}which can alter the internal pH and lead to increased fluidity of membrane and increased metabolism (Manjunath and

Table1. Average seminal plasma protein concentration (total and gelatin binding protein fraction) of Malabari bucks.

Animal No	Quality of the buck with respect to freezability of semen	Total seminal plasma protein concentration (mg/ml)	Concentration of gelatin binding fraction(mg/ml)	Proportion of gelatin binding fraction (%)
1 (n=6)	Good semen freezability	48.91 ± 3.91	27.16	55.54
2 (n=6)		64.90 ± 7.38	21.33	32.87
3 (n=6)		70.26 ± 2.97	27.99	39.84
Overall (n=18)		61.38 ± 6.13	25.49 ± 2.09[a]	42.75 ± 6.7
4 (n=6)	Poor semen freezability	52.44 ± 13.3	20.07	38.26
5 (n=6)		55.16 ± 2.06	18.14	32.88
6 (n=6)		59.31 ± 1.72	20.10	33.89
Overall (n=18)		55.64 ± 3.1	19.64 ± 0.64[b]	35.0 ± 1.65

Overall mean values with different superscripts within a column differ significantly (p<0.01).

Therien, 2002). Harshan *et al.* (2006) noted that high cryo damage was caused to buffalo epididymal spermatozoa which were treated with Fn2 type seminal plasma proteins.

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