PURIFICATION OF MAJOR FERTILITY-ASSOCIATED PROTEINS FROM SEMINAL FLUID OF VECHUR BULLS BY AFFINITY CHROMATOGRAPHY

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ABSTRACT

Bovine seminal plasma proteins (BSPs), is a family of threeacidic proteins, namely BSP-A1/-A2, BSP-A3 and BSP-30kDa, which form major fertility-associated proteins in bull seminal plasma. Present study was undertaken to purify homologous proteins from seminal plasma of Vechur bulls by affinity chromatography. For this, proteins in semen was precipitated by adding cold ethanol, dissolved in ammonium bicarbonate solution and lyophilized. The proteins were adsorbed to gelatin-agarose by affinity chromatography and then eluted by 8M urea in PBS. Absorbance of fractions was checked at 280 nm and protein containing fractions were pooled and proteins were precipitated. SDS-PAGEof gelatin-bound proteins revealed presence of three bands corresponding to molecular weight of BSP proteins. The results indicated the existence of gelatin binding proteins in Vechur bull seminal plasma and gelatin-agarose affinity chromatography was found to be reliable and effectivetool for purification of BSP proteins.

INTRODUCTION

Seminal plasma is an important reproductive secretion produced by male animals. It is rich in proteins which not only play role in pre-fertilization events occurring in sperms, like motility, capacitation, acrosome reaction, binding to oviduct epithelium, oocyte binding, etc., but also influence post-fertilization events like blocking polyspermy and early embryonic development.

A family of three acidic proteins, namely BSP-A1/-A2, BSP-A3 and BSP-30kDa, called as Bovine seminal plasma proteins (BSP proteins), occur in seminal plasma and account for 40 to 57 percent of proteins(Nauc and Manjunath, 2000).

BSP-A1 and BSP-A2 being glycoforms of same protein, they are regarded as single entity BSP-A1/A2 (Esch *et al*., 1983). The physiological roles of BSP proteins are well studied. At ejaculation, BSPs bind with choline phospholipids in sperm membrane,and remove (8 to 10 percent) cholesterol called 1st cholesterol efflux (Therien *et al*., 1998) and stabilize the membrane by limiting free movement of phospholipids. This inhibits premature acrosome reaction chieflyby blockingthe action of phospholipase A2(Manjunath *et al*., 1994).High density lipoprotein (HDL), along with heparin like glycosaminoglycans (GAG) present in the oviduct and/ follicular fluid remove sperm bound BSPs,while HDL cause 2nd cholesterol efflux (Therien *et al*., 1998). This elicits increase in membrane calcium permeability and intracellular pH leading to

capacitation, which is followed by acrosome reaction. BSP proteins also enable sperms to bind to oviductal epithelium (Gwathmey *et al*., 2006) for formation of sperm reservoir and maintaining motility.

However, BSPs are detrimental to sperms at higher concentration and/ at longer period of exposure that especially occursduring storage, wherein it causes continual cholesterol and phospholipid efflux decreasing the viability of sperms (Therien *et al*., 1998 and Therien *et al*., 1999). Low-density lipoproteins present in egg yolk based diluents are found to sequester BSP proteins thereby ameliorate their effects (Manjunath *et al*., 2002).

Homologs of BSP proteins are demonstrated installion, boar and buck but not so far in Vechur bulls. Hence this study is undertaken to standardize the technique gelatin–agarose affinity chromatography for purification of BSP homologs from Vechur bull seminal plasma.

MATERIALS AND METHODS

Materials

Semen from five Vechur bulls was obtained from Vechur farm, Vechur Cattle Conservation project, Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy. The following chemicals, glassware, commercial kits and equipment were used; Affigel-15(Bio-Rad, USA), Gelatin, Morpholino ethanesulphonic acid (MES), Sodium azide, Ethanolamine-hydrochloride, Tris-Hcl, Glycine, SDS and Urea (Himedia), Chromatography column: 300×10 mm (Borosil), Fraction collector (Model 2110), TGX: stain free fast cast kit, Low-range molecular weight protein standards and Mini-protean tetra cell (Bio-Rad, USA) and Lowry's protein estimation kit (Merck, Germany).

Methods

Coupling of gelatin to agarose and preparation of columns for affinity chromatography

As prescribed by Manjunath *et al*. (1987), contents of Affigel 15 were taken in muslin cloth todrain away the preservative liquid.25 mL gel slurry was washed with three bed volumes of cold (4^0 C) distilled water and was transferred to 50 mL falcon centrifuge tube. 25 mL gelatin solution (20 mg/mL in 0.1 M MES solution, pH 6.5) was added and kept for linear shaking at 25^0 C for 2 h. Two mL of 1 M ethanolamine-hydrochloride was added to neutralize any gelatin-unbound active esters, followed by an hour of linear shaking. After coupling reaction, the gel was transferred to chromatography glass column. Successive washing with 10 bed volumes of water, 8 M urea and PBS (pH 7.5) were carried out and the column was stored at room temperature by layering with 0.2 percent sodium azide.

Isolation of seminal plasma proteins from semen

Semen was collected in split ejaculates from single bull by artificial vagina method. As described by Manjunath (1984), the semen was centrifuged at 1000 ×*g* for 10 min.at 4^0C. The supernatant was aspirated and further centrifuged at 10,000 ×*g* for 10 min. at 4^0C to obtain seminal plasma. Nine volumes of cold (-20^0C) ethanol was added to seminal plasma and kept at 4^0 C for 90 min. with constant stirring to precipitate proteins,followed by centrifugation at 10,000×*g* for 10 minutes at 4^0 C to pellet the precipitate. The pellet was dissolved in 50 mM Ammonium bicarbonate and lyophilized (-84^0 C), to bestored in deep freezer (-20^0 C).

Gelatin-agarose affinity chromatography

As prescribed by Manjunath *et al*. (1987) and Boisvert*et al*. (2004), 'Phosphate buffered saline (PBS, pH 7.5) pre-equilibrated gelatin-agarose column' was loaded with 100 mg crude seminal plasma protein reconstituted with 3 mL PBS. Sample flow rate was maintained @

1 drop per 2 seconds. After sample has entered the column, stop cock was locked for 30 min. to allow proteins to interact with the column matrix. PBS and8 M urea in PBS were added in succession to wash out unadsorbed proteins and to elute bound proteins respectively. Fractions of 4 mL were collected during washing and elution, using fraction collector. The absorbance of fractions was checked at 280 nm and standard curve was plotted (Fig 1). The fractions under corresponding peaks were pooled: fraction A and fraction B (FA and FB), the protein content was in corresponding pool was estimated as per Lowry's protocol (Lowry *et al.,* 1951) and the proteins were precipitated, pelleted, dissolved in 50 mM Ammonium bicarbonate and lyophilized as per earlier procedure. Lyophilized FA and FB proteins were stored in deep freezer.

SDS-PAGE

Lyophilized crude seminal plasma proteins, unbound fraction (FA) and bound fraction (FB) were separated in 12% TGX stain free polyacrylamide gel using mini-protean tetra cell and molecular weight was estimated by referring to low-range molecular weight protein ladder.

RESULTS

Gelatin-agarose columns stored with preservative 0.2 percent sodium azide at room temperature could yield better results for8-10 times chromatography run in a period of 4-6 months without significant reduction in binding efficiency.

Figure 1 depicts the gelatin-agarose affinity chromatography profile of Vechur seminal plasma proteins. A and B represents the peaks under which the corresponding fractions contains unbound and bound proteins respectively. These fractions were separately pooled and protein content was estimated by Lowry's protocol (Lowry *et al*., 1951). Average protein content of FA and FB from all the bulls,as calculated by taking mean of individual values, was found to be 21.07 mg and 53 mg respectively. This indicates approximately 70 percent of the loaded proteins was recovered, out of which about 70 percent constituted bound proteins.

SDS-PAGE analysis of bound proteins (Fig 2) revealed presence of three protein bands, two of which showed molecular weight in the range of 15 to 17 kDa and one at 30 kDa.

DISCUSSION

Gelatin-agarose affinity chromatography is one of the standard techniques for isolation

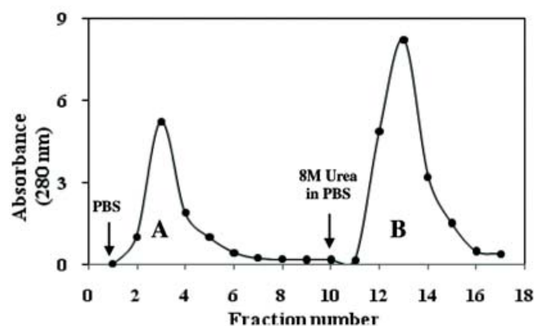

Fig.1 Gelatin-agarose affinity chromatography profile of Vechur Seminal plasma proteins; Graph plotted with absorbance (280 nm) against fraction number. Fractions under A and B peaks were pooled.

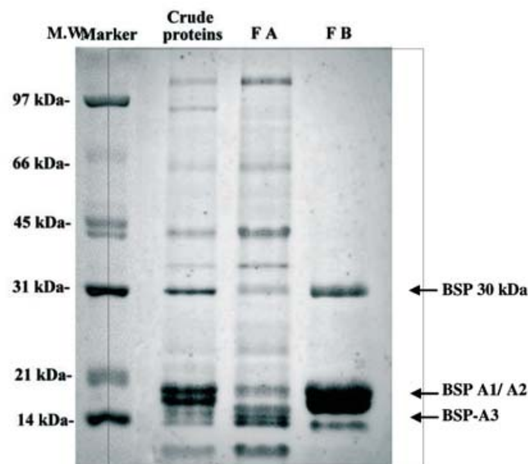

Fig. 2 SDS-PAGE of GAAC end products. 15 µg of crude seminal plasma proteins, 20 µg of FA (unbound proteins) and15 µg of FB (bound proteins) were reduced, denatured and electrophoresed in 12 % stain free gels. Low-range protein marker, diluted according to the supplier instructions, used to estimate molecular weight.

and purification of BSP proteins and it yielded good results with respect to Vechur bull seminal plasma in this study. The technique was proved to be simple and effective for isolation of BSP proteins.

Structure of BSP proteins show presence of two type-II domains which is identical to gelatin-binding domains of fibronectin (Esch *et al*., 1983; Seidah *et al*., 1987; Calvete*et al.*, 1996).This property was utilized here to purify BSP proteins as reported earlier byManjunath *et al.*(1987). Gelatin binding property of BSP homologs was also demonstrated in stallion, boar (Calvete *et al*., 1995; Calvete *et al*., 1997), goat (Michele *et al*., 2003) and bison (Boisvert *et al*., 2004). Thus it can be deduced that the gelatin-bound proteins obtained in our study may be homologs of BSP proteins with type-II domains.

Results of the study showed that, when 100 mg lyophilized crude seminal plasma proteins were loaded onto the column, after a series of elution with PBS and urea, about 70 percent of the proteins were recovered, out of which about 70% constituted gelatin-bound proteins. This indicates that gelatin binding proteins form major fraction in bull seminal plasma as reported earlier by Nauc and Manjunath (2000). SDS-PAGE of bound proteins showed 3 bands corresponding to molecular weight of BSP proteins;BSP-A1, BSP-A2, BSP-A3 and BSP-30kDa which have molecular weight of 16.5 kDa, 16 kDa, 15 kDa and 28 kDa respectively (Desnoyers *et al*., 1994). Since molecular weight of BSP-A1 and BSP-A2 differ only by 0.5 kDa, these two proteins may have migrated as single band during electrophoresis and also was found to be thickest of all the other bands..This observation coincides with the observations of Manjunath and Sairam (1987) that due to minute variation in molecular weight, BSP-A1 and A-2 migrated as single distinct band during electrophoresis, and these proteins alone constituted morethan 30% of seminal plasma proteins, resulting the band formed by them was intense. Hence the band may be composed of two proteins; BSP-A1 and BSP-A2.

To summarize, isolation of major fertility associated proteins from seminal fluid of Vechur bulls by gelatin-agarose affinity chromatography has been efficiently carried out. These proteins exhibited gelatin binding property, GAAC elution characteristics, abundance in seminal plasma and SDS-PAGE pattern similar to BSP proteins reported. Howeverfurther characterization shall be done by 2-D gel electrophoresis and amino acid sequencing study.

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