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Research Article

PARTIAL PURIFICATION AND IMMUNO-BIOCHEMICAL CHARACTERISATION OF FERTILITY ASSOCIATED PROTEIN OF KARAN FRIES BULL SEMINAL PLASMA

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ABSTRACT: The objective of the present study was detection, isolation, partial purification and immunobiochemical characterization of fertility associated protein in the seminal plasma of high prolific Karan fries bull. Seminal plasma of Karan Fries bull was partially purified by gel filtration chromatography and analyzed by 10% SDS-PAGE for their polypeptide profile. PAGE analysis revealed major band of 55 kDa, and 26 kDa. Hyperimmune serum was raised in rabbit against crude seminal plasma protein. Single precipitin line was observed in DID test when each of the partially purified 26 kDa and 55 kDa proteins were reacted with hyperimmune serum. These proteins were also found to be immunoreactive against hyperimmune serum in Western blot technique.

KEY WORDS: Karan Fries Bull, Seminal Plasma, 26 kDa, Fertility associated Protein, Immunodominant Protein.

INTRODUCTION

The development of bovine reproductive technologies such as artificial insemination has enabled reliable bull fertility measurements of individuals in a population of reproductively normal animals. These robust fertility data can be used as *in vivo* correlates for *in vitro* analyses of sperm and semen quality. The potential influence of seminal protein on male reproduction came to attention because of the studies showing that their expression is associated with breeding scores of dairy bulls (Killian *et al*., 1993, Cancel *et al*., 1997), beef bulls (Bellin *et al*., 1994**,** 1996, Parent *et al*., 1999**)** and Horses (Brandon *et al*., 1999)**.** Despite the relevance of those proteins, few have been identified (Cancel *et al*., 1997**;** Gerena *et al*., 1998; McCauley *et al*., 1999, 2001) and in most cases understanding of their function and relationships with fertility indexes is incomplete. Killian *et al*.(1993) reported the presence of four "fertility associated proteins" in the Holstein bull seminal plasma, two of which were later identified as prostaglandin D synthase (Gerena *et al*., 1998) and Osteopontin (Cancel *et al*., 1997). More detailed statistical

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analysis confirmed that the density of Osteopontin in 2D protein maps was responsible for 48% of the variation in non return rates of the bulls.

It has been established that there are fertility associated proteins in bovine and boar, such as 26 kDa and 55 kDa that are prevalent in seminal plasma from level of above average fertility. However no work was done on reproductively normal Karan Fries Bull seminal plasma proteins related to fertility.

In this background the present study is taken with following objectives:

1. Isolation and partial purification of the fertility associated protein from Karan Fries Bull seminal plasma.

2. Immuno-biochemical characterization of above partially purified protein.

MATERIALS AND METHODS

Collection of Semen: Fresh ejaculates were collected from ten reproductively normal Karan Fries Bull of 10-14 years old maintained in NDRI, Karnal, Haryana in the morning by using Artificial Vagina, and were immediately transported to the laboratory for further use.

Separation of seminal plasma: The seminal plasma was separated by centrifugation of semen at 3000 rpm for 30 min at 4^0C. The total protein concentrations of the seminal plasma were determined using bovine serum albumin as standard (Lowry *et al*., 1951). The samples were preserved at -20^0C for further analysis.

Preparation of partially purified seminal plasma antigen by gel filtration chromatography:

Sephacryl S-200 was packed into a column (60.0 cm x 1.5 cm) to isolate the polypeptides of crude seminal plasma antigen. The elution was carried out with equilibrating buffer containing PBS (pH 7.2), PMSF (0.03 mM) and 0.02% sodium azide at a flow rate of 20 ml per hour. Elutes were collected in 68 fractions of 3 ml each. The distribution of protein was monitored by taking the absorbance at 280 nm in a UV/VIS Spectrophotometer (SYSTRONICS-119). A graph was plotted by taking fraction numbers in the X axis and absorbances in the Y axis, which revealed a curve with major and minor peaks.

The protein fractions (PF) of seminal plasma of different peaks were pooled into seven parts, and named as P1 (PF 18,19,20,21,22 of first peak), P2 (PF 23,24,25 of ascending loop of second peak), P3 (PF 26,27,28,29,30,31 of descending loop of second peak), P4 (PF 32,33,34 of ascending loop of third peak), P5 (PF 35,36,37 of descending loop of third peak), P6 (PF 46,47,48,49 of fourth peak) and P7 (PF 50,51,52,53,54 of fifth peak).

The pooled protein fractions were concentrated by dialysis against sucrose using dialysis membrane (cut off value 12,000). The total protein concentrations of the 7 pooled fractions of seminal plasma were determined using bovine serum albumin as standard (Lowry *et al*., 1951). The concentrated peak fractions were then preserved at -20^0C with addition of protease inhibitors (0.03 mMPMSF).

Characterization of Polypeptide: The crude and partially purified samples were checked by one–dimensional SDS-PAGE in vertical mini slab-gel electrophoresis chamber (Genei, Bangalore, Karnataka, India) at a constant current of 9 mA for a period of 2.5 hours according to the method described by Laemmli (1970), with some modification under

denaturing and reducing condition using 10% polyacrylamide slab gel of 15 X 17 cm dimensions. Each lane of gel was loaded with 40 microgram of protein. After separation, gels were stained overnight in 0.25% Coomassie Brilliant Blue R-250 dye in methanol: acetic acid: water (50:10:40) and destained in methanol: acetic acid: water (30:10:60) destaining solution. Gels were finally preserved in 7% acetic acid solution.

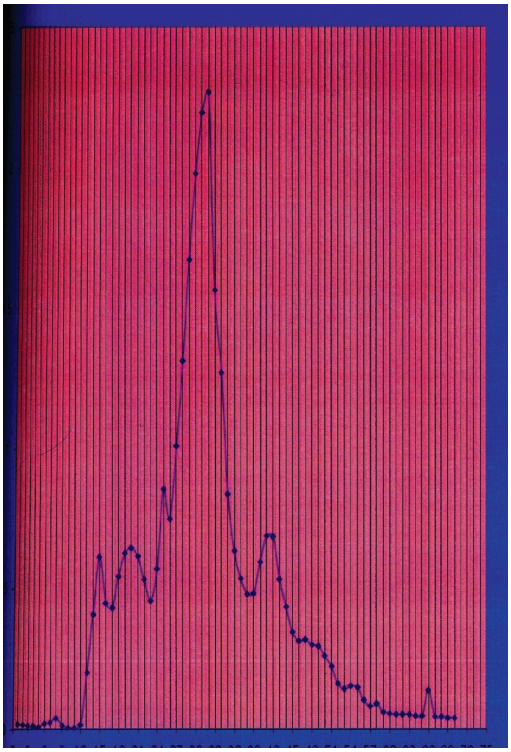
Immunobiochemicals analysis: The antibody against crude bull seminal plasma was raised in healthy New Zealand white rabbit with an average weight of 1.5 kg. The crude antigen was thoroughly mixed with equal volume of Freund's complete adjuvant (1:1) and a total of 1 ml was injected intramuscularly at three sites into the thigh muscle, and subcutaneously at three sites in the scapular region. Four booster doses of the same antigen emulsified with Freund's incomplete adjuvant (1:1) were given with subsequent increase in amount after 14 days interval following the first injection. Blood was collected from the heart of the rabbit 7 days after the last booster dose. Serum was obtained by centrifugation at 3000 rpm for 30 minutes at 4^0C and stored at -20^0C in aliquots for further use with sodium azide (0.02%) as preservative.

Double Immunodiffusion Test (DID): The DID test was performed according to the methodology of Hudson and Hay (1989) with some modifications. Agarose solution (1%) was prepared with PBS (pH 7.2), and a pinch of sodium azide was added to it. After solidification, the wells were filled with 20 µl of partially purified protein, 20 µl of hyperimmune serum raised against crude seminal plasma proteins and 20 µl of normal control serum in peripheral, central and peripheral wells respectively. The slides were then placed in humid chamber (37^0C) and incubated overnight. After washing the slide in PBS (pH 7.2), it was stained with Coomassie staining solution and destained with destaining solution.

Western Blot analysis: Western blot analysis was performed at room temperature (25^0C) after proteins were separated by SDS-PAGE (Laemmli 1970), and then the resulting proteins were electriblotted to nitrocellulose filter paper (Immobilon-P, Bedford, MA, USA) from gel according to the method of Towbin *et al*. (1979), Svoboda *et al*. (1985) using a Millipore Core, Bedford, MA, USA) at 50 mA for overnight at 4^0C. To minimize nonspecific protein binding, nitrocellulose sheets were incubated at 37^0C for 2 hours in PBS containing 5% BSA and 0.05% Tween 20. All additional washes and incubations were performed in PBS-Tween 20. After being washed, Nitrocellulose membrane was incubated with hyper immune serum raised against crude seminal plasma protein for 2 hours. After washing with PBS-Tween 20, Nitrocellulose membrane incubated with goat anti-rabbit horse radish peroxidase conjugate of 1:1000 dilutions in dilution buffer for 2 hours, followed by three times washing with PBS-Tween 20. Then the membrane was rinsed with substrate solution (10 ml Tris-HCL, 40 µl H_2O_2 and 2.5 mg Diaminobenzidine). The membrane was dipped into the distilled water to stop the reaction. Lastly, it was dried up and preserved.

RESULTS AND DISCUSSION

Analysis of seminal plasma protein: The protein concentration of pooled crude seminal



Graph No. 1- Purification of crude seminal plasma of Karan-Fries bull by Gel filtration chromatography on Sephacryl S- 200.

plasma was 18.8 mg/ml. 10% SDS-PAGE of crude seminal plasma, Stained with coomassie blue stain revealed several polypeptide bands. Seminal plasma proteins, prepared by gel filtration chromatography on Sephacryl S-200 with the flow rate of 20 ml/hour were resolved into five major peaks (Graph-1). The partially purified total protein concentration of pooled fractions of seminal plasma for P1, P2, P3, P4, P5, P6 and P7 were 5.44, 7.66, 5.39, 3.40, 5.58, 5.49, and 4.37 mg/ml, respectively. 10% SDS-PAGE of the different gel filtration fractions showed partially purified polypeptide bands when stained with coomassie blue stain (Fig.2).

Double Immunodiffusion Test: The double immunodiffusion test using partially purified seminal plasma protein with hyperimmune serum raised against crude seminal plasma protein showed single precipitin line in the slide (Fig.1) and thus confirmed its immunoreactive property. When this partially purified polypeptide was treated with normal serum, no precipitin line was evident.

Western blotting: The partially purified 55 kDa and 26 kDa seminal plasma protein was found to be immunoreactive against hyperimmune serum raised against crude seminal plasma in rabbit. (Fig-2).

The present study is the first to report the presence of 26 kDa protein in Karan Fries Bull seminal plasma that might have an important role in male fertility. In the present study, one dimensional SDS-PAGE of crude seminal plasma was carried out and several polypeptide bands were identified, which were similar to the finding of the Amann *et al.* (1987). When this crude seminal plasma protein was partially purified by gel filtration chromatography using Sephacryl S 200, four distinct major bands *viz.* 55 kDa, 26 kDa, 16 kDa and 14 kDa were obtained from different peaks, when resolved in 10% SDS-PAGE. This result is consistent with the findings of Killian *et al*. (1993), who reported the presence of fertility associated protein 26 kDa in Holstein bull seminal plasma, and Flowers (2001), who reported the presence of 26 kDa polypeptides in boar seminal plasma and their correlation with furrowing rate. It was beyond the scope of present study to evaluate the calving rate, but the mass activity, sperm motility and the morphology of spermatozoa indicated normal reproductive activity of the

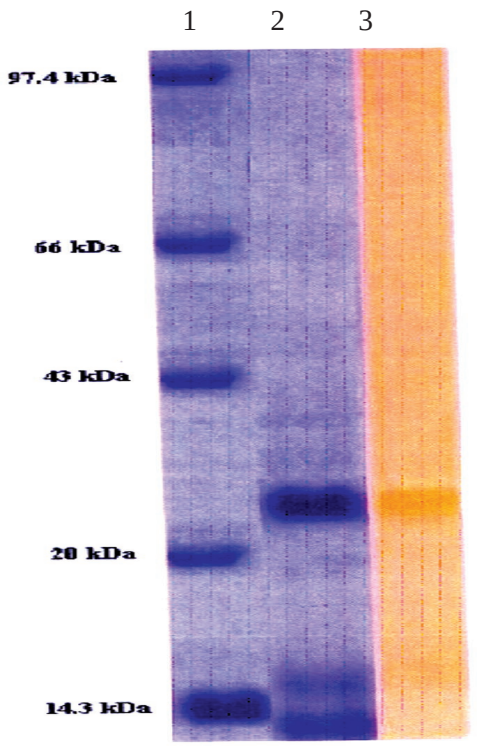


Fig. 1

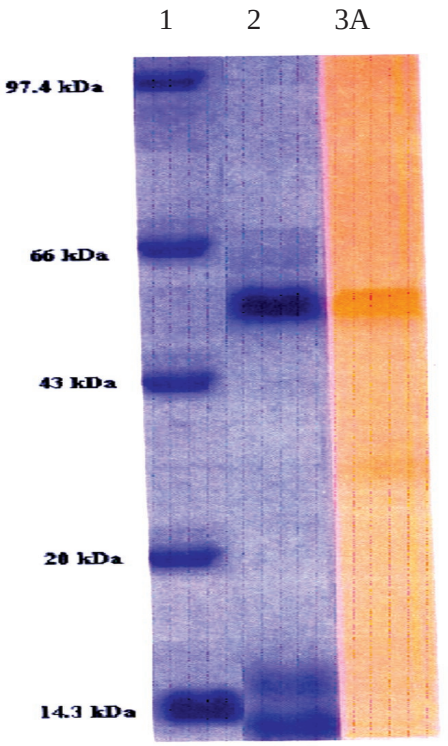


Fig. 2

Lane 1: Protein Molecular weight Standard
Lane 2: Partially purified fertility associated proteins
Lane 3: Immuno-reactive protein (26 kDa) in western blot analysis
Lane 3A: Immuno-reactive protein (55 kDa) in western blot analysis

Fig. 1& 2: SDS PAGE and Western Blot analysis of partially purified fertlity associated protein.

breeding bull. Polypeptide band of 26 kDa was predominant in the seminal plasma of Karan Fries Bull, when it was purified through gel filtration.

The double immune-diffusion test using partially purified seminal plasma protein of Karan Fries Bull with hyper-immune serum raised against crude seminal plasma protein showed single precipitin line in the slide and confirmed its immune-reactive property. When this partially purified polypeptide was treated with normal serum, no precipitin line was evident.

From the western blot analysis, it was clear that the hyper-immune serum raised against crude seminal plasma protein recognized the partially purified polypeptide of 55 kDa and 26 kDa. This is in support of the findings of Gerena

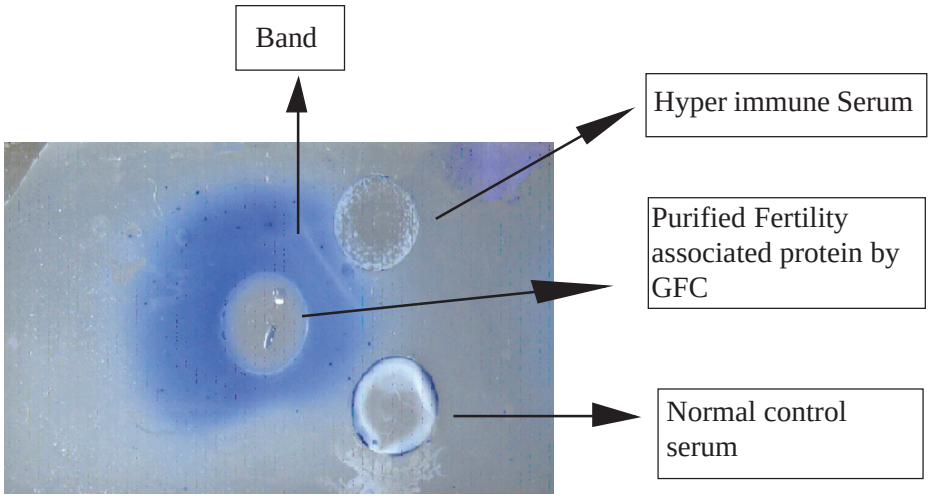


Fig. 3: Double immunodiffusion test.

et al. (1998, 2000), who recognized the 26 kDa polypeptide in the bull seminal plasma fluids by western blot analysis using column chromatography purified protein and in addition, the antiserum against this 26 kDa protein reacted with other Bull reproductive tract fluids like cauda epididymal fluid and rate testes fluid. The specific localization of this protein to the spermatids and Sertoli calls of the tests and to the primary cells of the epididymis, especially within the proximal caput segment, suggests that it plays an integral role in both the development and maturation of sperm (Gerena *et al*., 2000).

CONCLUSION

In the present study, Karan Fries bull seminal plasma was assessed for the detection of fertility associated protein along with their partial purification and immune-biochemical characterization. Two major fertility associated proteins of molecular weight of 55 kDa and 26 kDa were isolated from the seminal plasma of Karan Fries bull. These two highly significant proteins in respect of fertility of bull, as reported by the scientists, have been found to be immune-reactive by DID and Western Blot technique.

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