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Research Article

BOVINE PLASMA FIBRINOGEN AS MARKER IN CLINICAL AND SUB-CLINICAL MASTITIS

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ABSTRACT: Plasma samples were collected from healthy as well as clinical and sub-clinical mastitis affected cows from Barasat, West Bengal, India. Plasma samples, after ammonium sulphate precipitation, were dialyzed against several changes of PBS (pH 7.2) to remove the excess ammonium sulphate. Then plasma fibrinogens were purified by gel filtration chromatography on Sephacryl S-200 HR. SDS-PAGE (10%) of purified fibrinogen from plasma of healthy cow revealed polypeptide bands of 74, 67 and 57 kDa which represent the α (alpha), β (beta) and γ (gamma)- chains respectively. On the other hand, purified fibrinogen from plasma of sub-clinical and clinical mastitis affected cow revealed polypeptide bands of 73 (α-chain), 68 kDa (β-chain) and 72 (γ-chain), 68 kDa (β-chain) respectively. The SDS-PAGE analysis showed the absence of gamma (γ)- chain of fibrinogen in both the samples of sub-clinical and clinical mastitis positive cow. Single precipitin line was observed in double immunodiffusion test when purified fibrinogen from healthy, clinical and subclinical mastitis positive cows reacted with hyper immune sera raised in rabbit. No precipitin line was found against the normal control serum. These purified fibrinogens also showed cross reactivity against antibody raised in rabbit when analyzed by western blot technique.

Key words: Fibrinogen, Bovine, Mastitis.

INTRODUCTION

Fibrinogen is an important plasma protein and involved in homeostasis, providing a substrate for fibrin formation and in tissue repair, providing a matrix for the migration of inflammatory related cells (Thomas 2000). Fibrinogen specifically binds to CD11/CD18 integrins on the cell surface of migrated phagocytes, thereby triggering a cascade of intracellular signals that lead to enhancement of degranulation, phagocytosis, antibody-dependent cellular cytotoxicity and delay of apoptosis (Sitrin *et al.* 1998, Ruble *et al.* 2001). Another important function of fibrinogen is the formation of fibrin matrix that enables the movement of fibroblasts and other cells and stimulates their production during the healing of damaged tissue (Bakes and Illek 2006).

Fibrinogen (Fb) is a large, soluble glycoprotein. In 1959, Hall and Slayter produced electron microscopy images of fibrinogen revealing a rod-like molecule with three interconnected globules. Atomic force microscopyshowed a mixture of monomers, dimers and trimeric structures linked through globular interconnections (Marchant *et al.* 1997). The molecular weights of the individual chains are 66.5 kDa, (610 amino acids), 52 kDa (461 amino acids), and 46.5 kDa (411 amino acids) for the α, β, and γ -chain, respectively (Blomback and Blomback 1972, McDonagh *et al.* 1972).

Fibrinogen is an important acute phase protein (APP). The normal range of fibrinogen in adult bovine–plasma is 0.3-0.7 gm/dl. It increases in various inflammatory conditions of cattle, such as peritonitis, endocarditis, pericarditis, pneumonia, and nephritis. Fibrinogen is used in cattle as a reliable indicator of the presence of inflammation, bacterial infection or surgical trauma (Hirvonen *et al.* 1996, Cheryk *et al.* 1998, Hirvonen and Pyorala 1998).

There are only a limited number of haematological tests for the practicing veterinarian to diagnose bovine inflammatory diseases. In the field, the diagnostics are based on the case history and physical examination of the animal. Mastitis is a production disease of major importance in dairy farm. Cows with clinical signs of mastitis can be detected early by farmers. But, sub-clinical infection/mastitis may not be detected and remain untreated *i.e.*, fibrosis developed gradually in the affected

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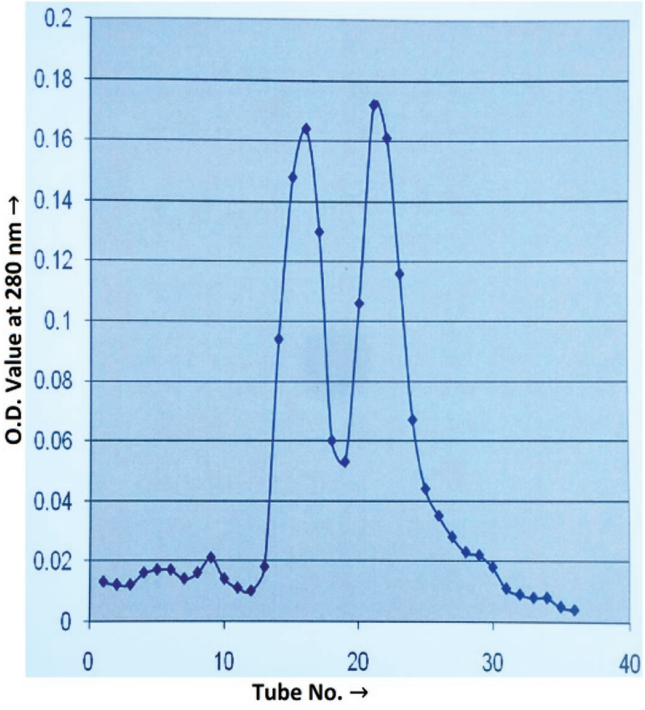


Graph 1. Purification of plasma fibrinogen from clinical mastitis positive cow by gel filtration chromatography on Sephacryl S-200.

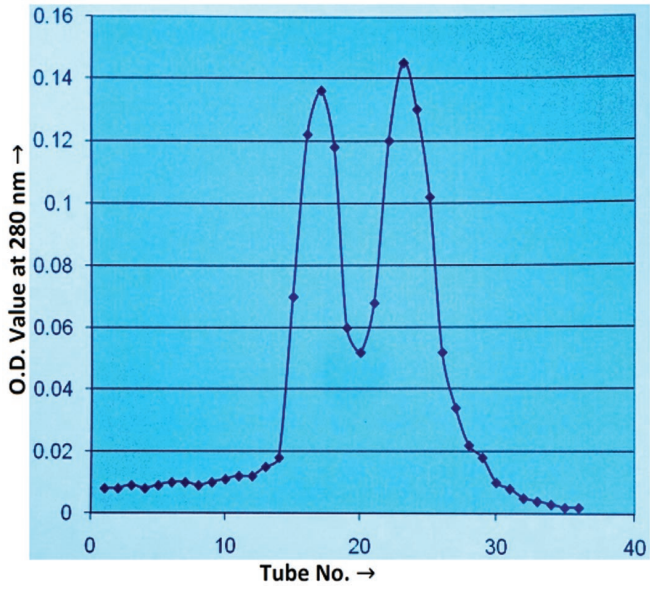
udder and it becomes non-functional completely.

Indicators of the inflammation can be determined from the plasma of infected cows and can be used for early detection of the infection. Plasma fibrinogen concentration may increase 2 to 10 fold during acute phase conditions (Tabrizi *et al.* 2008). Fibrinogen is the coagulation factor I, acute phase protein and is produced more rapidly than degraded during the inflammation. Although a number of acute phase proteins have already been characterized in the cow, little is known about fibrinogen status in bovine mastitis. The present study deals with isolation, purification and immune biochemical characterization of fibrinogen from bovine plasma and its relation with clinical and sub-clinical mastitis.

MATERIALS AND METHODS

After screening of clinical and sub-clinical mastitis by physical examination, Bromothymol Blue (BTB) card test as per Chanda *et al*. (1997) and Modified California Mastitis Test (MCMT) as per the standard procedures described by Schalm and Noorland (1957), the blood samples were collected from nine cows (three cows each in three groups, *viz*. healthy, sub-clinical mastitis and clinical mastitis positive cows) maintained in Ganganagar Cattle Rehabilitation Centre, Barasat, West Bengal.

The plasma was separated from collected blood samples by centrifuging at 2000 rpm for 10 min in the sterile plastic vials. Then the vials of the separated plasma



Graph 2. Purification of plasma fibrinogen from subclinical mastitis positive cow by gel filtration chromatography on Sephacryl S-200.

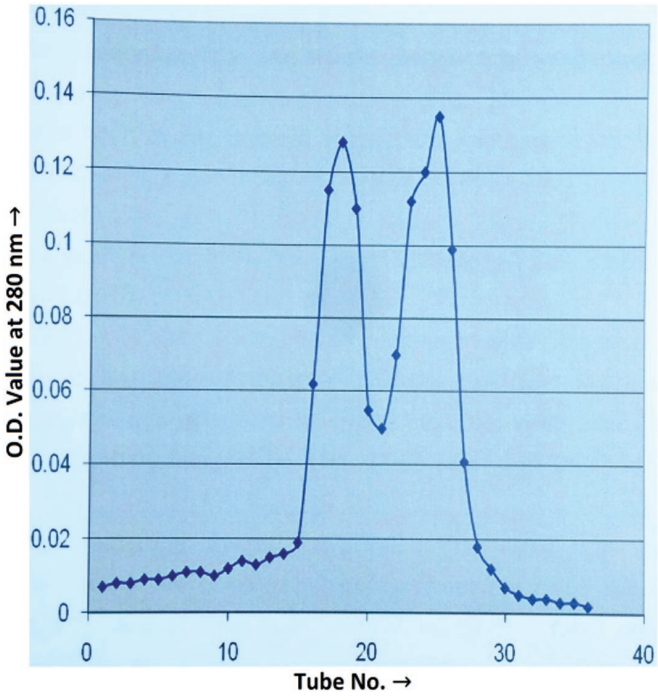
were kept in deep freeze in the laboratory.

For ammonium sulphate fractionation, 3 ml of plasma was mixed with 0.75 ml ammonium sulphate (20% saturated) separately. Then the vials were kept at 4^0C for 1.5 hour followed by centrifugation at 3000 rpm for 20 min and precipitates were separated. Then each precipitatewas dissolved in 3 ml of PBS. It was dialyzed against several changes of PBS (pH 7.2) for 24 hours at 4 ºC. This was done to remove the excess ammonium sulphate. Thus crude fibrinogen was obtained from clinical, sub-clinical mastitis and healthy cows (Parfentjev *et al.* 1953).

Protein concentration of plasma fibrinogen wasestimated by Lowry's method (Lowry *et al.* 1951) using UV-VIS Spectrophotometer (Systronics-119).

The precipitated fibrinogen samples were purified by gel filtration using Sephacryl S-200 HR (2 cm diameter and 42 cm in length). The protein concentrations of the two pooled fractions were determined by Lowry's method (Lowry *et al*. 1951). The concentrated peak fractions were then preserved at -20^0C in aliquots for further use. Then the two parts of pooled fractions of each sample were mixed together and average protein concentrations were determined.

The crude and purified samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in vertical slab gel electrophoresis (ATTO Corporation, Japan) as per method described by Laemmli (1970). Relative molecular weights (M_r) were determined by using protein markers (PMW–M, Bangalore Genei) containing phosphorylase (97.4 kDa), BSA (66 kDa), ova albumin (43 kDa), carbonic



Graph 3. Purification of plasma fibrinogen from healthy cow by gel filtration chromatography on Sephacryl S-200.

anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa) and analyzed by Gel Documentation System (Bio-Rad).

Antisera against crude plasma fibrinogen proteins of clinical mastitis positive cow was raised inhealthy New Zealand white rabbits (with an average weight of 1.5 kg) using Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA). Blood was collected from the rabbit 7 days after the third booster dose (each booster dose was given in weekly intervals) and serum was stored at -20^0 C in aliquots for further use with sodium azide (0.02%) as preservative.

The double immunodiffusion test (DID) was performed as per methods described by Hudson and Hay (1989).

For western blotting, the proteins were separated by 15% SDS-PAGE and then the resultant proteins were electroblotted to nitrocellulose filter paper (Immobilon-NC) from gel according to the method of Towbin *et al.* (1979).

RESULTS AND DISCUSSION

Isolation and purification of fibrinogen from bovine plasma

The protein concentration of the crude fibrinogen (*i.e.* not absolutely purified fibrinogen) samples after ammonium sulphate precipitation from three types of bovine plasma (healthy, sub-clinical mastitis and clinical mastitis positive cows) were 17.53 mg/ml, 21.07 mg/ml and 41.20 mg/ml respectively.

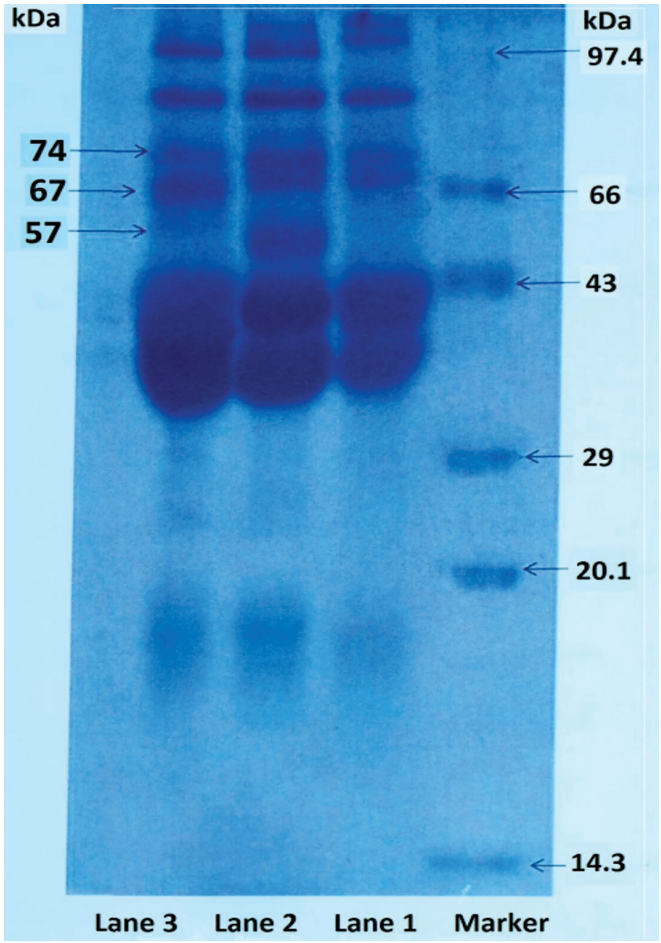


Fig. 1. 10% SDS-PAGE analysis of plasma fromhealthy (lane-3), clinical mastitis positive (lane-2) and subclinical mastitis positive (lane-1) cow.

Purified bovine plasma fibrinogen from sub-clinical, clinical mastitis positive and healthy cow was prepared by gel filtration chromatography on Sephacryl S-200 with the flow rate of 20ml/hr. The gel filtration experiment was carried out to know the difference in the concentration of plasma fibrinogen in three groups of animals under study. The graph, plotted by taking the absorbance of eluted fractions revealed a double bell shaped curve in which the proteins were resolved into two major peaks. First peak (Graph1) of clinical mastitis positive samples were pooled together into one part *i.e.* FCM1 (fractions no. 15, 16 and 17) which contain peak as well as upper ascending and descending part of the major peak and the fractions showing the second peak (Graph1) were pooled together into another part *i.e.* FCM2 (fractions no. 20, 21 and 22, 23) which contain peak as well as upper ascending and descending part of the major peak. First peak (Graph 2) of subclinical mastitis positive samples were pooled together into one part *i.e.* FSCM1 (fractions no. 16, 17 and 18) which contain peak as well as upper ascending and descending

part of the major peak and the fractions showing the second peak (Graph 2) were pooled together into another part *i.e.* FSCM2 (fractions no. 22, 23 and 24, 25) which contain peak as well as upper ascending and descending part of the major peak. Similarly, First peak (Graph 3) of samples from healthy cows were pooled together into one part *i.e.* FH1 (fractions no. 17, 18 and 19) which contain peak as well as upper ascending and descending part of the major peak and the fractions showing the second peak (Graph 3) were pooled together into another part *i.e.* FH2 (fractions no. 23, 24 and 25, 26) which contain peak as well as upper ascending and descending part of the major peak.

The protein concentrations of FCM1 and FCM2 were 1.51 mg/ml and 1.35 mg/ml; FSCM1 and FSCM2 were 0.813 mg/ml and 0.771 mg/ml; FH1 and FH2 were 0.787 and 0.745 mg/ml respectively. The two pooled fractions of each sample were concentrated against sucrose in dialysis bag (cut off value 12000). The concentrations of the fractions - FCM1 and FCM2 were 5.75 mg/ml and 6.53 mg/ml; FSCM1 and FSCM2 were 3.35 mg/ml 2.95 and mg/ml; FH1 and FH2 were 3.25 mg/ml and 2.85 mg/ml respectively. Then two parts of pooled fractions of each sample were mixed together and average protein concentrations were determined as 6.14 mg/ml (FCM), 3.15 mg/ml (FSCM) and 3.05 mg/ml (FH). Ekwumemgbo *et al.* (2010) isolated and purified bovine fibrinogen by cold ethanol precipitation and anion exchange chromatography which gave fibrinogen in pure form (98-99%), although sequencing was not done.

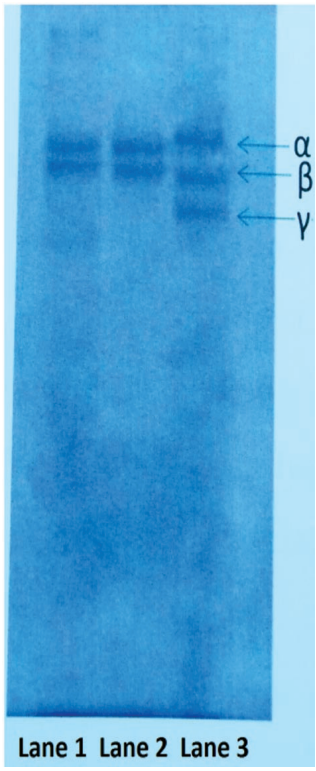


Fig. 2. 10% SDS-PAGE analysis of purified fibrinogen from subclinical mastitis positive (lane-1), clinical mastitis positive (lane-2) and healthy (lane-3) cow.

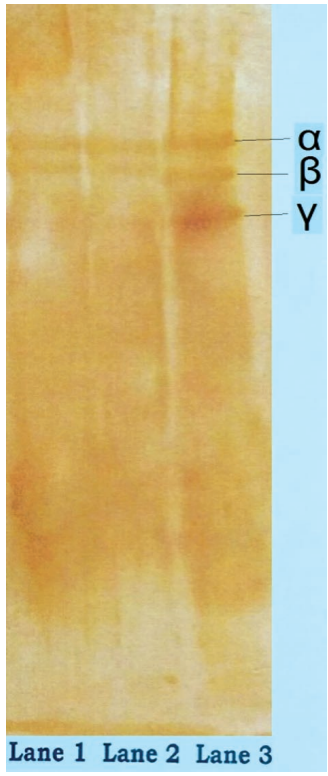


Fig. 3. Western blot analysis of plasma fibrinogen from subclinical mastitis positive (lane-1), clinical mastitis positive (lane-2) and healthy (lane-3) cow.

Biochemical characterization of partially purified bovine plasma fibrinogen

The purity of pooled fractions along with ammonium sulphate precipitated samples *i.e.* crude fibrinogens were checked by 10% SDS-PAGE. Plasma of healthy cow revealed some major polypeptide bands of 106, 100, 87, 74, 67, 57, 37, 19 and 18 kDa (Fig.1, Lane 3). Plasma of clinical mastitis positive cow revealed some major polypeptide bands of 106, 101, 95, 87, 72, 68, 51, 40, 35, 19 and 18 kDa (Fig.1, Lane 2). Plasma of sub-clinical mastitis positive cow revealed some major polypeptide bands of 107, 102, 86, 73, 68, 40, 36, 19 and 18 kDa (Fig. 1, Lane 1).

The molecular weights of purified fibrinogen from healthy cow revealed polypeptide bands of 74, 67, 57 kDa (Fig. 2, Lane 3) which represent the α (alpha), β (beta) and γ (gamma) – chains of fibrinogen, respectively. On the other hand, samples from clinical mastitis positive cow revealed polypeptide bands of 72 and 68 kDa (Fig. 2, Lane 2) and from sub-clinical mastitis positive cow revealed polypeptide bands of 73 and 68 kDa (Fig.2, Lane 1). Jennissen and Demiroglou (2006) isolated and purified the healthy bovine fibrinogen and analyzed in SDS-PAGE which revealed the polypeptide bands of 65.4 (α-chain), 61.9 (β-chain) and 53.3 kDa (γ-chain). Ekwumemgbo *et al.* (2010) also isolated and purified healthy bovine fibrinogen and analyzed the molecular weights by SDS-PAGE. The SDS-PAGE analysis showed the polypeptide bands of 63.1, 56.2 and 47.0 kDa which correspond to three chains α, β and γ of fibrinogen molecules, respectively. Results obtained in the present study are in consonance with the findings of Jennissen and Demiroglou (2006), Ekwumemgbo *et al.* (2010).

Double immuno diffusion test (DID)

Single precipitin line was observed in DID test in each case when purified fibrinogen from clinical mastitis, sub-clinical mastitis positive and healthy cow was reacted with the hyperimmune serum raised in rabbit. No precipitin line or band was found against the normal control serum. An incomplete round precipitin line was also observed in DID test when purified fibrinogen from clinical mastitis, sub-clinical mastitis positive cow and healthy cow were reacted with the hyper-immune serum,

but no precipitin line or band was found here also against the normal control serum. No reaction of hyper-immune serum against the normal control serum might be due to low concentration of plasma fibrinogen.

Detection of immunodominant polypeptide in western blotting

From the western blot analysis it was clear that the hyperimmune sera raised against purified plasma fibrinogen reacted with the polypeptide bands of 74, 67 and 57 kDa as α (alpha), β (beta) and γ (gamma) – chains of fibrinogen from plasma of healthy cow; the polypeptide bands of 73 and 68 kDa as α (alpha) and β (beta) – chains of fibrinogen from plasma of sub-clinical mastitis positive cow and the polypeptide bands of 72 and 68 kDa as α (alpha) and β (beta) – chains of fibrinogen from plasma of clinical mastitis positive cow (Fig. 3).

The alpha (α), beta (β) and gamma (γ)- chains of healthy bovine fibrinogen were immune reactive against rabbit sera; whereas only the alpha (α) and beta (β) - chains of subclinical and clinical mastitis positive bovine fibrinogen are immune reactive against rabbit sera respectively *i.e*., gamma (γ)- chain is absent in both of the mastitis cases.

CONCLUSIONS

From the present study, it may be concluded that - the plasma fibrinogen of healthy cows could be isolated and purified by salting out and gel filtration chromatography. The molecular weights of purified fibrinogen from healthy cows are 74 (α-chain), 67 (β-chain) and 57 kDa (γ-chain). In case of clinical and sub-clinical mastitis, the γ (gamma)- subunit of fibrinogen remains absent which may be considered as an important indication for early detection of mastitis. Therefore, research investigation may be conducted involving large number of clinical and sub-clinical mastitis cases in this direction.

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