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Isolation and Purification of C9 and Vitronectin from Goat Plasma

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ABSTRACT

Background: Vitronectin (Vn) is a multifunctional protein of blood and extracellular matrix. It binds to complement C9, which is involved in the terminal step of cytolysis including those of microbes. It hypothesized that Vn may bind to pore farming complement protein *i.e* C9 to modulate innate immunity and involved in immune evasion mechanism of bacterial pathogens like *staphylococcus aureus*.

Methods: The procedure allows the rapid, large-scale isolation of pure and haemolytically active Vn and C9 proteins from goat plasma by suitable ion-exchange and affinity chromatographic techniques.

Result: Vitronectin (Vn), a multifunctional protein of blood and the extracellular matrix has a mol.wt.of 78 kDa. Goat C9, the last component of complement, was purified in good yield by a combination of salt fractionation and ion-exchange chromatography. Approximately 1mg of protein can be obtained from 1 litre of the serum. The C9 was obtained has a mol.wt.of 66 kDa, determined by SDS/polyacrylamide-gel electrophoresis. No impurities were detected on gel electrophoresis and the Vn and C9 were confirmed by western blotting.

Key words: Affinity chromatography, Complement C9 protein, Ion exchange chromatography, SDS-PAGE, Vitronectin, Western blotting.

INTRODUCTION

The multifunctional glycoprotein vitronectin (Vn) that is found both in plasma and in the extracellular matrix (ECM) play important roles in homeostasis, cellular adhesion and in the regulation of the terminal pathway of complement and MAC (Bergmann *et al*., 2009; Bhakdi and Tranum-Jensen, 1981). Vitronectin exists as a 75-kDa protein in the ECM and as two truncated forms in plasma. These two forms, 65 and 10 kDa are held together by a disulfide bond. The N-terminal part of vitronectin is equivalent to Somatomedin B and binds plasminogen activator inhibitor-1 (Gechtman *et al*., 1997). This domain is followed by an Arg-Gly-Asp binding sequence that is important for the vitronectin-dependent movement and attachment of epithelial cells by interacting with several integrins including the $\alpha_v \beta_3$ integrin. Vitronectin also has three heparin sulphate-binding domains and binds plasminogen at its C-terminus (Suchitra *et al*., 2003). The importance of vitronectin-mediated interactions with bacteria was recently shown with pneumococci that utilize vitronectin and its integrin-binding capacity for adhesion and invasion of epithelial cells (Bergmann *et al*., 2009; Zipfel *et al*., 2013).

Microorganisms that are encountered daily in the life of a healthy animal cause disease occasionally. Most are detected and destroyed within minutes or hours by defence mechanisms that do not rely on the clonal expansion of antigen-specific lymphocytes (Dupuis *et al*., 1993). These are the mechanisms of innate immunity (Chauhan and Moore, 2006). The first part of the innate immune system that meets invaders such as bacteria is a group of proteins called the complement system. These proteins flow freely in the blood and can quickly reach the site of an invasion where they can react directly with antigens. When activated, the complement proteins can trigger complement cascade which results in the formation of membrane attack complex (Varela and Tomlinson, 2015).

The membrane attack sequence is the common cytolytic pathway of the classical and alternative pathways of complement and involves five plasma proteins, C5, C6, C7, C8 and C9 (Chauhan and Moore, 2006; Noris and Remuzzi, 2015). The complex constitutes the only known mechanism of blood plasma which is capable of impairing biological membranes. The damage to biological membranes commences with the formation of small pores at the stage of C5b-8 and large pores are formed after C9 binding (Bhakdi and Tranum-Jensen, 1981; Dupuis *et al*., 1993; Varela and Tomlinson, 2015). Lysis of bacteria or nucleated cells, which are relatively resistant to complement action, often requires C9 (Schreiber and Muller Eberhard, 1974; Varela and Tomlinson, 2015). Complement component C9 is a multi-domain protein that contains an N-terminal type-1 Thrombospondins (TSP) domain, a Low density lipoprotein-receptor class A repeat, several potential trans membrane

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(TM) regions and a C-terminal Epidermal growth factor-like domain. Hydropathy analysis of the sequence indicates the N-terminal half of C9 to be predominantly hydrophilic, while the C-terminal section is more hydrophobic. The amphipathic organisation of the primary structure is consistent with the known potential of polymerized C9 to penetrate lipid bilayers, causing the formation of trans membrane channels (Stanley *et al*., 1985). Studies on the mechanism of Staphylococcus resistance to complement-mediated killing reveal the involvement of extracellular matrix proteins like Vitronectin (Haixiang *et al*., 2006; Martin *et al*., 2006; Milis *et al*., 1993; Podack and Muller-Eberhard, 1980; Podack *et al*., 1984). Therefore, to understand the interaction between extracellular matrix proteins like Vn and complement C9 in animals, first, we should develop a method for isolation and purification of these proteins from the blood. Thus, this study was aimed to establish a combination of appropriate chromatographic techniques for isolation and purification of Vn and C9 proteins from the goat blood.

MATERIALS AND METHODS

Goat blood was collected from local abattoir near Indian Veterinary Research Institute, Bareilly, Uttar Pradesh as soon as possible after the animals were slaughtered and transported to the laboratory in an ice cold condition. The study was carried out during the period from July 2015 to January 2016 in the Division of Animal Biochemistry, IVRI, Izatnagar.

Materials

DEAE-Spharose, Sepharose 4B, Heparin Sepharose, 3, 3′ aminobenzidine (DAB), Phenylmethylsulfonyl fluoride (PMSF), Polyethylene Glycol-4000, Tween 20, acrylamide, bis-acrylamide, Tris, protease inhibitor cocktail were procured from Sigma-Aldrich (USA), Rabbit anti-human C9 polyclonal antibody and a secondary antibody conjugated to horse radish peroxidase from (Santacruz, Dallas, TX, U.S.A), protein molecular weight markers (Bio-Rad), Dithiothreitol (DTT) and urea were brought from Biogene (USA).Nitrocellulose membranes were from MDI (India). All other reagents were of high-grade purity.

Methods

Purification of complement C9 from goat plasma

C9 was purified from goat serum following published protocols with modifications. In brief, goat serum with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was centrifuged at 3,000 g, at 4°C for 10 min to pellet aggregates formed during storage. All subsequent steps were carried out at 4°C and solutions contained 0.5 mM PMSF and 0.03% sodium azide.

Twenty-four milli litres of 1M $BaCl_2$ was added in batches to 500 mL of serum, with constant stirring for 15 min. The suspension was centrifuged for 10 min at 27000 g. The precipitate was discarded and 250 ml of 21% polyethylene glycol 4000 (PEG 4000) was added to the supernatant with constant stirring. After an hr, the suspension was centrifuged at 27000 g for 15 min. The precipitate was discarded. To the supernatant, 105 g of solid PEG was added with stirring and kept for 60 min. It was centrifuged as before and the pellet was saved and dissolved in C9 solubilizing buffer (20 m M Tris-Cl buffer (pH 7.4), 0.03% sodium azide and 0.5 mM PMSF).

The protein solution was dialyzed against 20 mM Tris-Cl buffer (pH7.4), 0.03% sodium azide and centrifuged at 27000 g for 10 min at 4°C. The supernatant was collected and fractionated on a DEAE-Sepharose column. The unbound fraction was saved and the bound proteins were eluted by the stepwise increase of NaCl from 25 mM to 500 mM. Fractions of 3ml were collected and analyzed on a 12% poly acrylamide gel. Protein bands were visualized after Coomassie brilliant blue staining (CBB) and the presence of C9 protein in the fractions was confirmed by Western blot using rabbit anti-human C9 polyclonal antibody (H-210 rabbit polyclonal Ig-G Santacruz, Dallas, TX, U.S.A). The secondary antibody was goat anti-rabbit IgG-HRP conjugate. The C9 protein, obtained after DEAE-Sepharose chromatography, was dialyzed against (20 mM Tris-Cl buffer pH 7.4, 0.03% sodium azide) and loaded to Vn-Sepharose column. The unbound fraction was collected, the column was washed with excess buffer and the bound proteins were eluted by passing 1M NaCl. The presence of C9 protein in the eluted fractions was confirmed by Western blot.

Purification of vitronectin from goat blood

Goat Vn was purified essentially as described previously (Mahawar and Joshi, 2008) and is briefly outlined below. Goat plasma with 1 mM PMSF was fractionated on Sepharose 4B and Heparin-Sepharose columns sequentially in the presence of 20 mM sodium phosphate, pH 7.4, containing 110 mM NaCl. Urea (6 M) and dithiothreitol (10 mM) was added to the adsorbed plasma and kept at room temperature (25°C) for 3 hr and then fractionated on a Heparin- Sepharose column containing urea. The column-bound Vn was eluted by increasing the NaCl concentration to 1 M. Fractions were analyzed for the presence of Vn by western blot and those containing Vn were dialyzed against PBS.

RESULTS AND DISCUSSION

Purification of Vn from goat plasma

Vn purified in the presence of urea is referred to as denatured Vn. SDS-PAGE analysis of denatured Vn showed two bands of ~ 81 kDa (Fig 1). Besides, a doublet of > 250 kDa, at the top of the separating gel was distinct and may constitute a multimeric form of Vn. These bands reacted with rabbit anti-goat Vn anti-serum in Western blot (Fig 2). These results are in agreement with previous findings (Mahawar and Joshi, 2008; Yatohgo *et al*., 1988).

Purification of C9 from goat plasma

C9 was purified by a combination of salt/PEG fractionation and anion-exchange chromatography followed by separation on a Vn-Sepharose column. The recovered protein had an apparent size of 66 kDa in SDS-gel (Fig 3). In addition, a

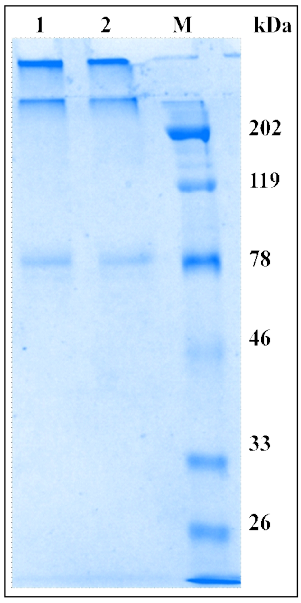

Fig 1: Coomassie Brilliant Blue-stained goat Vn.

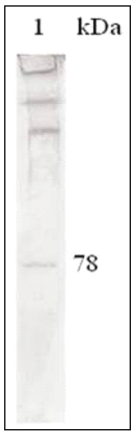

Fig 2: Western blot analysis for expression of goat Vn.

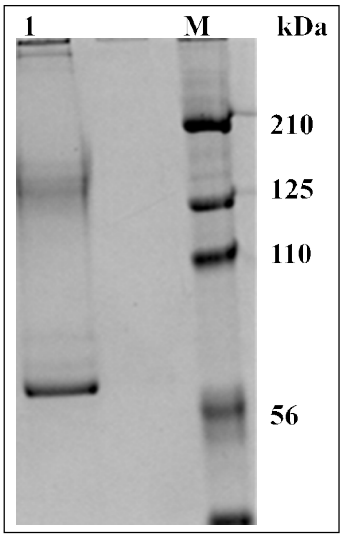

Fig 3: Coomassie Brilliant Blue-stained goat C9.

band of ~180 kDa and high molecular weight multimers were also observed and may represent aggregated products as the C9 tends to polymerize. The fine resolution and degree of purity of C9 complement component obtained in the first chromato-graphic step utilizing DEAE-Sepharose ion exchange cellulose were further purified through Vitronectin-Sepharose chromatography as shown in Fig 3. It has been shown by several investigators that C9 complement protein is present with albumin on weak anion exchange celluloses (Borsos and Rapp, 1965; Eisenschenk *et al*., 1992; Nilsson *et al*., 1966; Nilsson and Muller-Eberhard, 1965; Tamura and Shimada, 1971), presumably a result of their similar molecular structures. It has been reported that C9 complement protein which has very similar physicochemical properties with other complement proteins consequently also difficult to resolve using anion exchange chromatography (Podack *et al*., 1979; Nilsson and Muller-Eberhard, 1965). The resolution of C9 protein is possible when Vn-Sepharose is utilized as described here. Furthermore, the C9 post-DEAE and Vn-Sepharose preparations were determined to be 20% and 86% pure, respectively, as judged by SDS-PAGE. Thus, we have developed a methodology which provides pure complement component C9 in high yield from

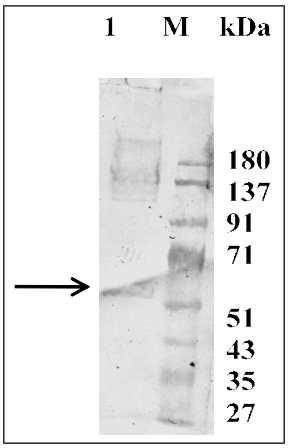

Fig 4: Western blot analysis for expression of goat C9.

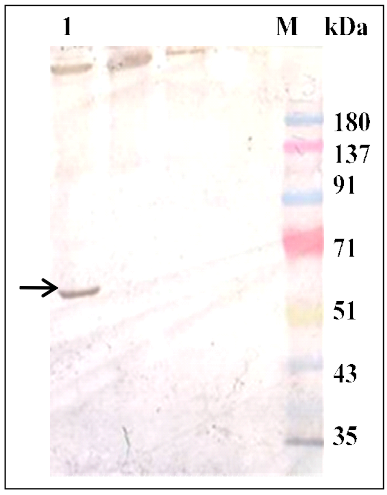

Fig 5: Western blot analysis for expression of bovine C9.

a single serum pool. The availability of this protocol should facilitate further studies of the interaction and biological function of the C9 complement component with other proteins.

Evaluation of complement C9 in goat plasma

The authenticity of the C9 protein is based on the following facts. First, the protocol used for goat C9 isolation was based on the methods developed for human and bovine C9 protein. Second, goat C9 reacted with rabbit anti-human C9 polyclonal antibody (Fig 4). This antibody also stained control lane having bovine C9 protein (Fig 5). Third, bovine and human C9 have a similar size, ~66 kDa. Furthermore, the sequence similarity of C9 among these species is quite high, bovine and human share ~81% identity whereas goat and human C9 have ~69% similarity.

CONCLUSION

For proper understanding of the immune evasion mechanism of gram-positive bacteria like *S.aureus* in animals it is necessary to study the host factors like interaction between Vn and Complement C9. The methods described in this article could be used for purification of these proteins in large quantity with high efficiency for generating different recombinant fragments of these proteins (like N-terminal, Middle fragment, C-terminal) using a suitable prokaryotic expression vector to determine the binding region of Vn to the C9.

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