ISOLATION AND PURIFICATION OF GOAT COMPLEMENT COMPONENT C9

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Abstract: A procedure for the isolation of complement (C) protein C9 from goat is described. The procedure allows the rapid, large-scale isolation of pure and haemolytically active protein. Goat C9, the last component of complement, was purified in good yield by a combination of salt fractionation and ion-exchange chromatography. Approximately 1mg can be obtained from 1 litre of serum. The C9 was obtained has a mol.wt.of 66,000 kDa, determined by SDS/polyacrylamide-gel electrophoresis. Goat C9 is haemo-lytically active glycoprotein and the purified protein in addition to the 66 kDa, a band of ~180 kDa and high molecular weight multimers were also observed and may represent aggregated products as the C9 has a tendency to polymerize. No impurities were detected either on gel electrophoresis.

Keywords: Complement component C9,Goat,DEAE-Sepharose chromatography, SDS-PAGE.

Introduction: Microorganisms that are encountered daily in the life of a healthy animal cause disease only occasionally. Most are detected and destroyed within minutes or hours by defense mechanisms that do not rely on the clonal expansion of antigen-specific lymphocytes. These are the mechanisms of innate immunity. 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 - molecules that the body recognizes as foreign substances. When activated, the complement proteins can trigger complement cascade which results in the formation of membrane attack complex. 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 (3,12).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 (1,4,19). Lysis of bacteria or nucleated cells, which are relatively resistant to complement action, often requires C9 (15,19). Complement component C9 is a multi-domain protein that contains an N-terminal type-1 TSP domain, an LDL-receptor class A repeat, a number of potential transmembrane (TM) regions and a C-terminal EGF-like domain. Hydropathy analysis of the sequence indicates the N-terminal half of C9 to be predominantly hydrophilic in character, while the C-terminal section is more hydrophobic. The amphipathic organisation of the primary structure is consistent with the known potential of polymerised C9 to penetrate lipid bilayers, causing the formation of transmembrane channels (16).Understanding the importance of complement C9 and lack of information of C9 in animals especially in goats,a procedure for isolation and purification of C9 was described .

Materials and Methods

Materials: DEAE-Sepharose,Sepharose-4B,Heparin-Sepharose,3,3'aminobenzidine(DAB), PMSF,Tween-20,acrylamide,bis-acrylamide,Tris,protease inhibitor cocktail were procured from Sigma-Aldrich (USA),secondary antibody conjugated to horse radish peroxidase, protein molecular weight markers from Biorad, IPTG, DTT and urea were brought from Biogene (USA).Nitrocellulose membranes were from MDI (India).All other reagents were of high-grade purity. Purification of Complement C9 from goat plasma C9 was purified from goat serum following published protocols (5) with modifications. In brief, goat serum with 0.5mM phenylmethylsulfonyl fluoride (PMSF) was centrifuged at 3,000g, at 4°C for 10 min to pellet aggregates formed during storage. All subsequent steps were carried out at 4° C and solutions contained 0.5mM PMSF and 0.03% sodium azide. Twenty –four millilitres 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 27000g.The precipitate were discarded and 250 ml of 21% polyethylene glycol 4000(PEG 4000) was added to the supernatant with constant stirring. After an h, the suspension was centrifuged at 27000g for 15 min.The precipitate was discarded. To the supernatant, 105g 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 solubilising buffer (20m M Tris-Cl buffer (pH7.4), 0.03% sodium azide, and 0.5mM PMSF). The protein solution was dialyzed against 20mM Tris Cl buffer (pH7.4), 0.03% sodium azide and centrifuged at 27000g 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 stepwise increase of NaCl from 25mM to 500mM. Fractions of 3ml were collected and analyzed on a 12% poly acrylamide gel.

Protein bands were visualized after Coomassie brilliant blue staining 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 (20mM Tris-Cl buffer pH7.4, 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.

Results: 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.1). In addition, a band of ~180 kDa and high molecular weight multimers were also observed and may represent aggregated products as the C9 has a tendency to polymerize.**Evaluation of Complement C9 in goat plasma**: The authenticity of 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.2). This antibody also stained control lane having bovine C9 protein (Fig.3). Third, bovine and human C9 have a similar size, ~66 kDa .Furthermore, sequence similarity of C9 among these species is quite high; bovine and human share ~81 % identity whereas goat and human C9 have ~69 % similarity.

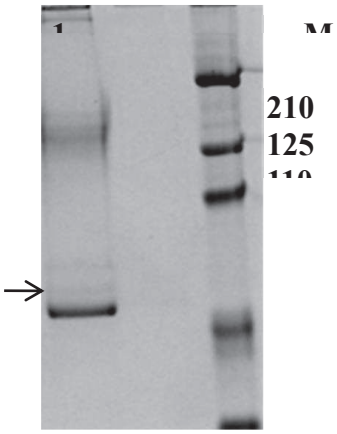

Fig 1. CBB-stained goat C9

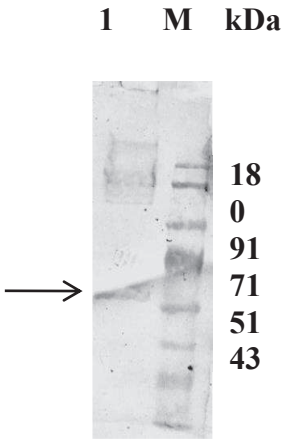

Fig 2.Western blot of goat C9

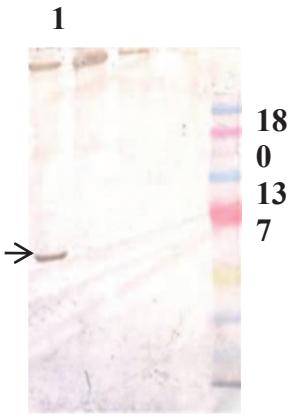

Fig 3.Western blot of bovine C9

Discussion: In this report we described a preparative method for the isolation and purification of C9 component of complement from one large pool of goat serum in which the purified product is characterized functionally and immunochemically (not shown here).The fine resolution and degree of purity of C9 complement component obtained in the first chromato-graphic step utilizing DEAE-Sepharose ion exchange cellulose was further purified through vitronecti-Sepahrose chromatography as shown in Fig.1. It has been shown by several investigators that C9 complement protein is present with albumin on weak anion exchange celluloses (2, 17, 9, 10, 6), presumably a result of their similar molecular structures (11). It has been reported that C9 complement protein which have very similar physicochemical properties with other complement proteins consequently also difficult to resolve using anion exchange chromatography (13,14,8). 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 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.

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