Three step purification of C1q by DNA precipitation, ion exchange and lectin affinity chromatography

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SUMMARY The difficulties associated with the isolation of pure C1q in sufficient amounts are reflected by the substantial number of isolation procedures, which are being published. The two major problems are a low yield and contaminating immunoglobulins. In addition, some isolation protocols appear to produce C1q contaminated with an inhibitor (C1q-INH).

The present isolation protocol involves precipitation of C1q by DNA, chromatography using Sephadex QAE A 50 followed by Con A affinity chromatography. By this combination of purification steps maximal advantage was taken of the cationic properties and high carbohydrate content of the C1q molecule. The yield was 1-2 mg C1q per 100 ml serum. The isolated C1q was free of any demonstrable contaminants as demonstrated by Ouchterlony double diffusion and polyacrylamide gel electrophoresis.

Serum C1q is a basic, high molecular weight glycoprotein (MW 410 000 pI 10) which occurs as a part of a three molecular complex consisting of C1q, C1r, and C1s.¹⁻³ Originally C1q, or the 11 S component, was described as a thermolabile protein capable of precipitating aggregated gammaglobulin.⁴ Since then a number of methods for the demonstration and isolation of circulating immune complexes have been introduced, many employing the interaction between C1q and the Fc fragment of immunoglobulin.^{5,6}

The complex function and structure of C1q and the various interactions of this molecule have lately been subject to intense investigation.⁷⁻¹¹ Usually the interest has been restricted to the properties of the globular head region of C1q, recently it has been shown, however, that the binding of C1q to blood mononuclear cells is mediated by the collagenous portion of the molecule⁷ and that binding of C1q to fibronectin takes place by a similar mechanism.^{12,13}

Functional, structural and biochemical studies usually require purified proteins. For these reasons there has been an increased need for isolated and functionally intact C1q. Although various methods for the purification of C1q have been described, the isolation is often associated with problems, especially low yield and contaminating immunoglobulins.

In this work DNA-precipitated C1q¹⁴ was further purified successfully using a combination of ion exchange and affinity chromatography. The purified protein was biologically functioning and free of contaminating immunoglobulins demonstrable by means of SDS-polyacrylamide gel electrophoresis and Ouchterlony gel precipitation methods. All the reagents and materials used in the C1q isolation protocol are commercially available.

Material and methods

SERA

Human plasma was defibrinated by adding 1 mM CaCl₂ and 85 µl thrombin (1000 IU/ml in 50% wt/wt glycin; Topostasin, Hoffman la Roche Ltd, Basel, Switzerland). The fibrinogen was removed by centrifugation and Ca⁺⁺ ions were precipitated by adding 200 mg sodium oxalate per 100 ml serum.

IMMUNODIFFUSION METHODS

Quantification of IgG, IgM as well as C1q was carried out using radial immunodiffusion plates (Behringwerke AG, Marburg, GFR). Ouchterlony double diffusion was performed in 1% agarose (Seakem Agarose, Marin Colloids Inc, USA) in barbital-acetate buffer, pH 8.2. Immunoelectrophoresis was carried out in 0.9% agarose, TRIS-barbital buffer, pH 8.6 u = 0.04. For the demonstration of C1q, immunoelectrophoresis was

performed in 0.1 M phosphate buffer, pH 7.5.² Rabbit antisera against human serum proteins, IgM, IgG and C1q were from Behringwerke and Dako Immunoglobulins (Copenhagen, Denmark).

PROTEIN ANALYSIS

Protein concentrations were measured with the Folin phenol reagent¹⁵ with 0.1% sodium dodecyl sulphate (SDS) in the reaction mixture. Polyacrylamide gel electrophoresis (PAGE) was performed using 8% slab gels containing SDS¹⁶ and stained with Coomassie blue.¹⁷ C1q was iodinated by the chloramine T method¹⁸ and labelled protein was studied by fluorography (Kodak X-omat film, USA).

DNA PRECIPITATION

Precipitation of C1q from serum was performed essentially as described previously.¹⁴ Briefly, serum was dialysed against a 0.025 M Veronal, 0.01 M ethylenediaminetetraacetate (EDTA) buffer, pH 8.6. Then 25 µg deoxyribonucleic acid (Calf Thymus DNA, Worthington Biochem Corp, Freehold, New Jersey, USA) per ml sera was added. The precipitate was collected by centrifugation and washed four times in 0.025 M Veronal buffer, pH 8.6 solubilised in a 0.05 M phosphate buffer (pH 6.9–7.0) containing 0.05 M NaCl 3 mM MgCl₂. To this, 100 µg DNase I (Worthington Biochem Corp, Freehold, New Jersey, USA) was added. After the precipitate had dissolved the mixture was dialysed against the phosphate buffer for 12 h. Insoluble aggregates were removed by centrifugation at 30 000 rpm for 30 min (Spinco SW 50, 1 rotor).

CHROMATOGRAPHY

Dry Sephadex QAE- A-50 (Pharmacia, Uppsala, Sweden) was dissolved in a buffer containing 0.01 M TRIS-HCl and 0.1 M NaCl; pH 7.5. After complete swelling, the gel was packed in columns possessing volumes about 10 times those of the sample. C1q containing fractions obtained from the ion exchange were pooled and applied on a Con-A Sepharose column (Pharmacia, Uppsala, Sweden) in a 0.01 M Veronal, 0.5 M NaCl, 0.001 M CaCl₂ buffer, pH 8.0 (based on the observation by Conradie *et al*¹⁹). After exhaustive washing the elution was carried out by making the elution buffer 0.5 M in α-methylmannopyranoside (Calbiochem AG, Switzerland). Before application, all samples were dialysed overnight against the gel buffer.

IMMUNOFLUORESCENCE MICROSCOPY

Antibody-independent binding of C1q to cytoplasmic filaments and cell nuclei of fetal human skin were used as an assay for functionally intact C1q.^{8,20}

Frozen sections were fixed for 15 min in 3.5% paraformaldehyde and treated with 0.1% of the non-ionic detergent NP 40 (BDH, Poole, England). Samples to be tested were incubated for 30 min at room temperature and subsequently stained with FITC-conjugated rabbit antihuman C1q (Behringwerke AG, Marburg GFR, molar fluorescein:protein (F:P) ratio = 1.7). For demonstration of bound immunoglobulins we used a FITC-conjugated sheep antihuman Ig (Polyvalent) conjugate F:P ratio 3:2 (NBL, Stockholm, Sweden). All washings were carried out in phosphate-buffered saline. The sections

Fig. 1 SDS polyacrylamide gel electrophoresis of reduced and alkylated C1q. Slot a: fluorography of purified ¹²⁵I-labelled C1q shows labelling of subunit C. Slot b: purified C1q, the 3 subunits, A, B and C can be distinguished. Slot c: phosphorylase (94 K), bovine serum albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), soya bean trypsin inhibitor (20 K). b and c were stained with Coomassie blue.

Fig. 2 Immunoelectrophoresis of isolated C1q. The cathode is to the left. Anti C1q: rabbit antihuman C1q, anti HSP: rabbit antihuman serum protein.

Fig. 3 Binding of C1q to fetal human skin.

were examined in a Zeiss standard microscope (epi-illuminator IV FL, Osram HBO 50 W high pressure mercury lamp).

Results

In all experiments the supernatant obtained after DNase digestion contained C1q which could be demonstrated in immunoelectrophoresis, double- and radial-immunodiffusion. The relative C1q content was 45–50%, one main contaminant was the DNase enzyme. C1q was found to elute in the void volume through the ion exchanger, no DNase was present and the relative C1q concentration rose to about 90%. From the lectin column the molecule was eluted when the elution buffer was made 0.5 M in α -methylmannopyranoside. In PAGE the purified C1q protein preparation appeared, under reducing conditions, as three distinct bands. In fluorography of the iodinated C1q preparation the label was almost entirely found in the C-chain (Fig. 1). The purified protein diffused poorly in agar gel, but

could be analysed using agarose gels. In immunoelectrophoresis, the purified C1q gave only one cathodic arc and showed a reaction of identity against antihuman serum protein and antiC1q (Fig. 2). Immunoelectrophoresis and Ouchterlony double diffusion assays of the purified C1q preparation (250 μ g/ml) did not reveal any contaminants when tested against antihuman serum protein antiserum. Additional double diffusion tests for purity were performed using C1q in concentrations ranging from 0.1–1.0 mg/ml against anti-IgG and anti-IgM. Also in these assays the C1q preparation was found to be free of contaminants. The immunofluorescence assay for complement binding was found to be a rapid and sensitive method for identification of C1q from different column fractions (Fig. 3) (5–10 μ g/C1q/ml still detectable). No contamination immunoglobulins were detected. The purified protein also precipitated aggregated γ -globulin in agarose gel and IgG-coated latex particles (data not shown).

Discussion

In this study DNA-precipitated C1q was successfully isolated using a combination of ion exchange and affinity chromatography. Being a strongly cationic protein, C1q was, in contrast to many other serum proteins, able to move through the QAE ion exchange column. By absorbing C1q to the immobilised Concanavallin A, it was possible to remove contaminating IgG, since this protein has a very poor affinity to the lectin.^{21,22} Any interaction between C1q and immunoglobulins was minimised by the high ionic strength of the buffer.¹ According to recent work this step would also remove a serum inhibitor for C1q.¹⁹ The purified protein moved cathodally in electrophoresis and reacted with antiC1q. Thus, the protein was identified as C1q.^{1–3, 23–25} We were not able, however, to demonstrate the reported anodal arc seen in immunoelectrophoresis.²⁶ Furthermore, when reduced and alkylated, it was possible to demonstrate the three peptide chains (A, B and G) of C1q in SDS-PAGE.^{24,25} During the purification, concentration by ultrafiltration was avoided, all buffer changes were performed by dialysis and C1q samples were stored at -70°C . At this temperature the final preparation was stable for at least five months.

The purified C1q reacted with cell nuclei and intermediate filaments similarly as serum C1q when foetal skin was used as substrate in the immunofluorescence assay.⁸ The binding of C1q not only to nuclei but also to the filaments appearing as "microfibrils" in the tissue sections suggests that

C1q is functionally active and capable of generating the classical pathway C3 convertase.^{20,27}

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