Isolation and Characterisation of C_1q from Human Serum*

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SUMMARY **The subcomponent C_1q, the first component of complement, was purified to homogeneity from human serum by affinity chromatography. The serum was euglobulin-precipitated at an alkaline pH; the reconstituted precipitate was chromatographed in rabbit anti-human C_1q covalently coupled to cyanogen-bromide-activated Sepharose-4B and also to Sepharose rabbit anti-normal human serum (C_1q depleted), C_1q was eluted by 1 M NaCl in the former while the flow-through of the latter was C_1q which was rechromatographed on a goat anti-human IgG Sepharose-4B column for removal of contaminating IgG.**

Final yields of C_1q ranged from 40 per cent to 70 per cent with a 420-to 490-fold purification of protein based on recovery of haemolytic activity. These preparations were free of contaminating serum proteins as judged by PAGE, SDS-PAGE and immuno-chemical criteria. However, the final C_1q preparation might have been contaminated with undetected C_1q inhibitor.

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Human C_1q is an unusual protein consisting of six triplet-stranded sub-units, each with a globular head that binds immunoglobulin and a collagen-like fibrillar tail that reacts with C_1r and C_1s.[1] It is of considerable interest because of its biological importance in initiating complement activation, the physical characterisation of C_1q, its interaction with C_1r and C_1s and with immunoglobulin. Also, C_1q has been used for the detection of circulating immune complexes in a wide variety of diseases[2,3] because it has the advantage of being a natural recognition protein for immune complexes by the classical complement system. For such studies, highly purified and biologically active C_1q is an essential prerequisite.

Various methods of C_1q isolation and purification utilise one or more of its chemical or biological properties. These include its minimal solubility in low ionic strength buffer,[4,5] reversible binding to heat-aggregated IgG,[6,8] precipitation with DNA[7] and reversible binding to solid phase human gammaglobulin with gel permeation and/or ion exchange chromatography.[8-12] In this communication, a rapid reproducible affinity column chromatography procedure for isolation of highly purified C1q is described.

MATERIALS AND METHODS

Serum: Human volunteers were bled; clotting proceeded at 37°C for two to three hours. The serum was obtained by wringing the clot and by centrifugation.

Reagents: Cyanogen bromide (CNBr)-activated Sepharose-4B and concanavalin-A were procured from Pharmacia Fine Chemicals, Uppsala, Sweden and Nobel Agar from Difco Laboratories, Detroit, MI, U.S.A. Acrylamide, N, N[1] methylene bis-acrylamide, Coomassie brilliant blue, sodium dodecyl sulphate (SDS), dithiothreitol (DTT), iodoacetamide, ethylene diamine tetra-acetic acid (EDTA), and methyl-D-glucopyranoside (MGP) were purchased from Sigma Chemicals, St. Louis, MO, U.S.A. Ammonium persulphate and N,N,N,N-tetramethylene diamine (TEMED) were purchased from Eastman, Rochester, N.Y., U.S.A. Antisera to IgG, IgM and IgA were produced locally and anti-C_1q was obtained from Cappel Laboratories, Malvern, PA, U.S.A.

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Immunoadsorbents

Rabbit anti-human C_1q RAH C_1q) was coupled to Sepharose-4B, 6g of CNBr-activated Sepharose-4B was washed in a Buchner funnel with 2 litres of 1 mM HCl followed by 250 ml of coupling buffer (carbonate-bicarbonate buffer, pH 8.9). Monospecific anti C_1q [2 ml (14.2 mg/ml)] was added and stirred gently for two hours at 37°C and subsequently washed with 0.1 M glycine-HCl, pH 2.5, followed by 0.01 M of phosphate buffered saline (PBS, pH 7.5). A protein determination was done on the filtrate and the amount of protein coupled was calculated to be 11-13 mg/ml Sepharose 4B.

Human IgG was purified by precipitation from sera with a 50 per cent final concentration of the ammonium sulfate and dialysed against PBS. The dialysate was centrifuged and applied to a DEAE cellulose column (2x20 cm) pre-equillibrated in PBS. Elution was carried out by increasing the salt concentration (0.005-0.2 M). Fractions containing IgG were pooled, concentrated and dialysed. Human IgG (HIgG) was coupled to CNBr-activated Sepharose-4B as described above and the amount of protein coupled was calculated to be 15-20 mg IgG/ml of Sepharose-4B. Fresh, pooled, normal human serum was made 10 mM in EDTA and applied to the HIgG Sepharose-4B column. The flow-through fractions were pooled and employed as C_1q-depleted (C_1qD) normal human serum (NHS).

Goat anti-human IgG (GAH IgG) and rabbit anti-NHS (C_1q-depleted) (RANHS) were prepared by immunising subjects with five subcutaneous injections of pure IgG and NHS (300-400 μg each) emulsified in complete Freund's adjuvant at weekly intervals. Two booster doses in incomplete Freund's adjuvant were given thereafter. Serum was obtained one week after the final injection and precipitated with 33% cold saturated ammonium sulphate at 4°C. After equillibrating the mixture for 30 minutes, the precipitate was collected by centrifugation (4,000 rpm, 10 min.), dissolved in PBS, exhaustively dialysed against PBS and coupled to CNBr-activated Sepharose-4B as described above.

The concanavalin A (Con A) Sepharose-4B column was equillibrated with 0.01 M veronal buffer, pH 8.0, containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$ (Con A buffer). Purified protein was applied and the column run at 22°C with a flow rate of 5 ml/hr; 1 ml fractions were collected. After the first peak, the solution was made at 10 per cent strength with alpha-methyl-D-glucopyranoside and bound protein was eluted.

Isolation procedure

Serum was titrated to pH 8.7-8.9, made 5 mM with EDTA and dialysed against 10 mM ethylene diamine-HCl, pH 8.8, for 20 hours in a ratio of 20:1 (buffer/serum). The mildly tubid solution was centrifuged at 4,000 g for 40 minutes to obtain C_1q-rich euglobulin precipitate and dissolved in 0.5 M NaCl containing 0.002 M EDTA, pH 7.4. Delipidification was carried out by centrifuging at 30,000 g for 90 minutes.

The $RAHC_1q$ and RANHS columns were washed prior to use with 0.1 M glycine-HCl, pH 2.5, followed by coupling buffer and finally with PBS containing 0.01 M EDTA. Euglobulin precipitate was applied and the column kept at 4°C overnight. $RAHC_1q$ was washed with 0.15 M NaCl buffer at a flow rate of 40 ml/hr until the absorbance at 280 nm of the flow-through material was less than 0.01. Elution of bound C_1q was carried out with 1 M NaCl in PBS containing 0.05 M EDTA (pH 7.5); fractions were concentrated and dialysed against PBS.

From the RAHNS column, the C_1q was in flow-through fractions, the bound material was eluted with 40% ethylene glycol. Fractions containing C_1q were rechromatographed on an RAH IgG Sepharose column to get rid of contaminating IgG. The RAH IgG column was prewashed with 0.01 M PBS containing 1.0 M NaCl, 0.05 M EDTA (pH 7.5).

Characterisation of C_1q

Immunoelectrophroesis (IEP) was carried out as described by Williams[13] using 1.5% agar in barbital buffer, pH 8.6. Electrophoresis was performed for six hours under a constant current of 5-10 mA per immunoframe. IgG, IgA and IgM were characterised in various fractions by Ouchterlony double diffusion[14] and quantified by radial immuno diffusion[15] in 1.5% agarose containing 0.01 M PO_4, 1.0 M NaCl (pH 7.5) and specific antibody. C_1q was detected by rocket immunoelectrophoresis as described by Laurell[16] and concentration was determined spectrophotometrically[17] using E_{280} 1% of 0.682. Protein concentration was determined by Folin phenol reagent[18] using bovine serum albumin standard.

C_1q haemolytic assay

The haemolytic activity of C_1q was assayed according to Kolb *et al.*[8] 20μl of appropriate dilution of the sample were added to a mixture containing 300 μl of GVB (isotonic veronal buffered saline containing 0.15 M $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% gelatin), 40 μl of C_1q-depleted serum (20 μl of 1 M $CaCl_2$ and 1 M $MgCl_2$ stock ml C_1qD) and 200 μl of EA ($5x10^7$/ml). The tubes were incubated at 37°C for 45 minutes at which time 1 ml of ice-cold GVB was added. After centrifugation, the OD of the supernatant at 412 nm was determined. The effective molecules were calculated according to Borsos and coworkers[19]

Polyacrylamide gel electrophoresis

PAGE was conducted using 7.5% gel in 0.1 M tris glycine buffer as described by Davis.[20] SDS–PAGE

Table 1. Purification of C_1q from human serum.

Fraction	Total protein	Total activity units*	Sp. activity units/mg protein	Yield	Purification (fold)	C_1q	Sp. activity mg C_1q/mg protein	Purification (fold)
Normal human serum	1,448	24,504	16.9	100.00		.5568	.0004	–
Euglobulin ppt	7.6	18,044	2,374.2	73.60	140	.5136	.0676	169
RAH C_1q column	0.031	16,935	8,338.2	69.10	492	.3867	.1904	476
RANHS column	2.025	14,657	7,238.0	41.00	427	.3395	.1677	419

*One unit of activity is defined as $1x10^8$ effective molecules.
+C_1q determined spectrophotometrically using $E^{1\%}_{280}$ of 0.682.

was conducted according to Weber and Osborn.[21] For electrophoresis under non-reducing conditions, iodoacetamide (0.05 M) was added while reduced conditions were achieved by the addition of dithiothreclol (10 mM) without iodoacetamide. After electrophoresis, the gels were fixed in 10% TCA for half an hour and stained with 0.25% Coomassie brilliant blue in methanol, acetic acid and water (10:1:9 v/v) for 20 hours and destained by 7.5% acetic acid containing 5% methanol.

RESULTS

Table 1 shows C_1q recovery and the purification factor at different steps. Euglobulin precipitation by 5 mM ethylenediamine, pH 8.8 after titrating the sera containing 5 mM EDTA to pH 8.7 to 8.9, resulted in a 140-fold increase in specific activity with 74 per cent recovery of C_1q. Under these conditions, immunoglobulin contaminates (Table 2) which were away from their isoelectric point (IpH) at this pH remained soluble, whereas C_1q which was close to its IpH was precipitated from the sera.

Chromatography of euglobulin material on the $RAHC_1q$ column (Fig. 1) and RAHNS column (Fig. 2) resulted in a highly enriched preparation of C_1q with a 492- and 427-fold purification of protein along with a total recovery of 69 per cent and 41 per cent respectively from the two columns (Table 1). The recovery of C_1q from the RAHNS column was very poor which may have been due to the presence of trace amounts of anti-C_1q which we were unable to detect otherwise in our anti-NHS.

Table 2 Characterisation of other immunoglobulins during purification.

Procedure	IgG	IgA	IgH	C_1q*
NHS	+++	+	++	+
RAH C_1q column flow-through	+	–	+	±
RAH C_1q column eluant	–	–	–	+++
RAH NS column flow-through	+	–	±	+++
RAH NS column eluant	++	–	+	±

*C_1q assayed by rocket immunoelectrophoresis.

PAGE analysis of the pooled fractions containing C_1q gave a single band (Fig. 3b) in the case of the $RAHC_1q$ column while two bands (Fig. 3C) were seen when eluted from the RAHNS column due to the contaminating IgG (Table 2), elimination of the IgG from the C_1q preparation was efficiently accomplished on the GAH IgG column at 1.0 M NaCl (Fig. 4). PAGE of the eluted protein gave only one band corresponding to IgG (Fig. 3d). In high salt concentrations, C_1q is unable to bind to solid-phase GAHIgG, whereas the antibody activity for human IgG is not affected. Thus, C_1q passes in the flow-throughs whereas contaminating human IgG is retained.

Similarly, we have used 1 M NaCl for SRID or rocket IEP, since C_1q precipitates with preformed immune complexes or heat-aggregated IgG[7] and antibodies dissociated in semi-solid agar at 56°C, may cause heat aggregation giving false positive results due to C_1q precipitation.

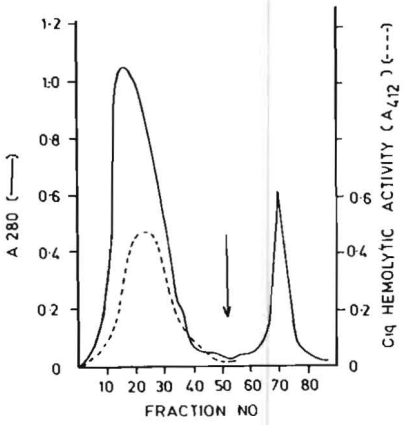

Fig. 1 Rabbit anti-human C_1q Sepharose-4B affinity column chromatography. Flow rate was 20 ml/h & 1 ml fractions were collected. The arrow indicates elution with 1 M NaCl in 0.01 M phosphate buffer containing 0.05 M EDTA, pH 7.5. Fractions were assayed for protein and C_1q haemolytic activity.

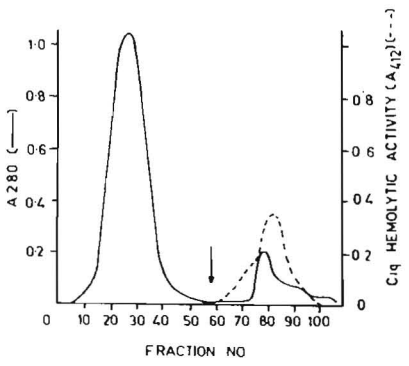

Fig. 2 Rabbit anti-normal human serum (C_1q depleted) Sepharose-4B column chromatography. The column was pre-equilibrated with 1 M NaCl in 0.01 M phosphate buffer containing EDTA (0.01 M). Bound protein was eluted with (arrow) 40% ethylene glycol. The flow-through material contained a small amount of IgG.

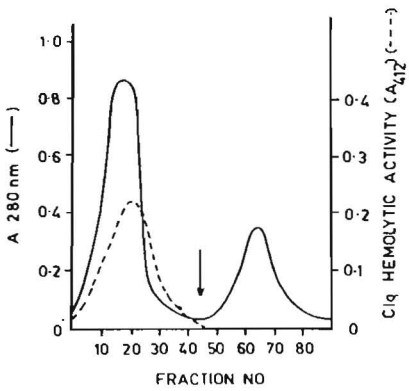

Fig. 4 Rabbit anti-human IgG Sepharose-4B chromatography of flow-through of the RAHNS column. The arrow indicates the start of 40% ethylene glycol. The eluted peak was demonstrated to be IgG.

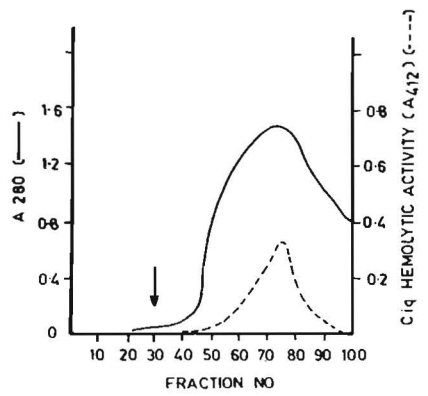

Fig. 6 Con A Sepharose-4B column was equilibrated with Con-A buffer; flow rate was 5 ml/hr with 1 ml fractions collected. Increase in absorbant at 280 nm is due to presence of αMGP in the elution buffer.

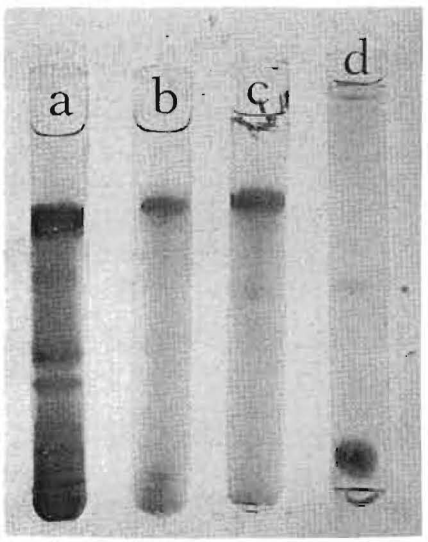

Fig. 3 Polyacrylamide gel electrophoresis of (a) euglobulin precipitate (b) rabbit anti-human C_1q Sepharose-4B eluate, (c) rabbit anti-human normal serum flow-through and (d) eluted peak of rabbit anti-human IgG Sepharose-4B column.

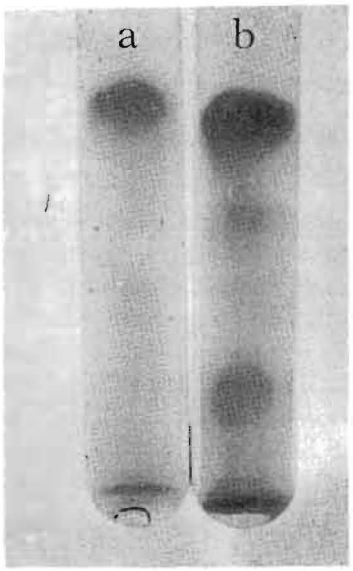

Fig. 5 SDS-Polyacrylamide gel electrophoresis. Electrophoresis of purified C_1q was performed on 9% acrylamide gel under (a) non-reducing and (b) reducing (DTT) conditions.

Ouchterlony analysis of the C_1q preparation revealed a heavy precipitation line with anti-C_1q, but no precipitation line was evident when tested against anti-IgM, IgA, IgG or NHS. IEP analysis showed one line with anti-NHS which was immunologically identical to that produced by anti-human C_1q. No IgG or IgM was detected. SDS-PAGE analysis (Fig. 5) under non-reducing conditions produced two bands (Fig. 5a) while three bands were observed (Fig. 5b) upon reduction with DTT. The C_1q preparation at this step was practically homogeneous although no functional test was performed to determine the presence of other complement components. These results suggest no contamination since no other band was detected on PAGE even at a high level of loading. The yield of C_1q was approximately 14-20 mg from 1 litre of serum.

This preparation was then applied to a Con A Sepharose column (Fig. 6) as described by Kolb, Kolb and Padack,[8] but no separation was achieved and we could not detect any inhibitor in the flow-through fractions.

DISCUSSION

Since C_1q has minimal solubility in low ionic strength buffers and since EDTA dissociates the C1 macromolecule into its constituents, this property has been utilised by many workers to prepare euglobulin precipitate[4,5] but this results in considerable immunoglobulin contamination. The preparation of euglobulin at an alkaline pH after titrating 5 mM EDTA serum to pH 8.7-8.9 resulted in complete C_1q precipitation, whereas immunoglobulin contamination, which is away from IpHs under these conditions, remains soluble. Also, for euglobulin prepared at this pH, lipid contamination is almost nil.

Pohl and coworkers[11] have reported a 505-fold purification with 52 per cent yield of C_1q from human plasma, whereas Kolb *et al*[8] have reported a 833-fold purification of protein with only a 40 per cent yield by a method involving Sepharose human IgG and Biogel A-5 M. In the present report, yields of 41-69 per cent of the C_1q present in serum were obtained with a 420-490-fold purification factor. These values are similar to those obtained by Angello and coworkers[7] and somewhat lower than those reported by Tenner and coworkers.[12]

In addition to high yields of active C_1q, the present method is comparatively simple and rapid. Affinity chromatography on Sepharose RAHC_1q or Sepharose RAHNS (C_1qD) followed by Sepharose GAH IgG column yielded homogeneous C_1q. However, the yields of C_1q were much better on Sepharose RAH C_1q so it may be concluded that this matrix can be efficiently used for larger yields of pure C_1q. Neither IgM nor IgG, frequent contaminants of C_1q preparations, was detectable in the final preparation by immunochemical analysis or by PAGE. However, considerable loss of C_1q occurred during the concentration of various fractions, in part due to the formation of irreversible insoluble aggregates.

Conradie *et al*[22] have described a proteoglycan inhibitor in human serum. Silvestri *et al*[23] have characterised it as a naturally occurring chondroitin sulphate proteoglycan inhibitor (MW 800,000 to several million) which can form complexes with C_1q in human serum and which can co-precipitate with C_1q during purification procedures. Kolb *et al*[8] have separated this inhibitor from C_1q by the Con A affinity column in the presence of 0.65 M NaCl. We failed to achieve this separation even in the presence of 1 M NaCl. This can be explained on the basis that C_1q, also being a proteoglycan, will bind to a Con A bed along with an inhibitor and that it co-elutes along with C_1q inhibitor by 10 per cent MGP.

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