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Isolation of the C3 complement component and its C3d subunit from IY-1 fraction of Cohn's fractionation of human plasma

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Abstract

C3 complement component and its C3d subunit were isolated from the IY-1 Cohn's fraction, which is the waste of industrially produced albumin and immunoglobulins. The first step was the fractionation of precipitate IY-1 by polyethylene glycol (PEG) 4000 to a final concentration of 16% PEG. The precipitate formed was separated by centrifugation. The supernatant contained the C3d subunit of C3, and the redissolved 16% PEG precipitate contained the C3 component. Then the supernatant and the dissolved precipitate were subjected to anion-exchange chromatography on DEAE-Toyopearl 650 M. In the last step fractions containing C3 and C3d concentrated by ultrafiltration were chromatographed on Sephacryl S-200.

1. Introduction

The third complement component C3 is an important participant in the complement and immune system, having a concentration in human serum of about 1.2–1.5 mg/ml (1% of plasma protein) [1–4]. The molecule is composed of two polypeptide chains with molecular masses of 115 000 (α-chain) and 75 000 (β-chain) [5] associated both by a single disulfide bond and non-covalent forces [6]. Activation of both classical and alternative pathway convertases (C4b2a and C3bBb, respectively) leads to proteolytic cleavage of C3 to C3a and C3b [5–8]. The C3b, M_r 185000 fragment, containing a thioester between cystein 1010 and glutamine 1014 on the α-chain of the molecule [6–8], immediately reacts with many membranes and surfaces or with water by a transesterification reaction [9,10]. C3b, which is bound to the membranes, may then lead to lysis of the cell or stimulate a variety of immunological phenomena, such as opsonization and phagocytosis [3,11,12]. Fluid-phase C3b is cleaved by regulatory proteins, factors H and I (C3b inactivator), forming an intermediate molecule, C3bi. Then C3bi may react with a variety of enzymes such as trypsin, kallikrein, elastase, urokinase, plasmin or cathepsin G forming C3c (M_r 130 000–140 000) and C3d (M_r 40 000) fragments [13,14], the latter having a disulfide bond. Several proteolytic cleavage products of C3 (C3b, C3bi, C3d) have been reported to influence humoral and cell-mediated immune responses [1–3, 11–14]. Now it is possible to use C3 complement component in complex therapeutic preparations. Inoue [15]

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established that transfusion of the C3-rich fraction should have beneficial effects in surgical patients (post-operative, septic, cirrhotic) with severely depleted plasma opsonic activity, in addition to replacing plasma. In vivo administration of the C3-rich fraction remarkably improved the clearance of bacteria and the survival rate of opsonin-depleted animals with experimental *Escherichia coli* peritonitis.

Because of the important role of C3 in the complement and immune system, many efforts have been made to improve the methods of purification of this protein as the original methods were both time consuming and low in yield [16,17]. One improvement was made by Molenaar et al. [18] by using affinity adsorbents made from antiserum. A method based on fractionation of plasma with sodium sulphate and precipitation of euglobulin fractions following DEAE-cellulose and hydroxyapatite chromatography was described by Harrison and Lachmann [19]. However, a true breakthrough in C3 purification was made by Tack and Prahl [5], who described a method of purifying C3 from fresh human plasma by employing an initial fractionation with polyethylene glycol, followed by sequential depletion of plasminogen by affinity adsorbents, chromatography on DEA-cellulose, gel permeation on agarose and batch adsorption/desorption on hydroxyapatite with a 30% yield.

Methods for the preparation of C3d from plasma were described by Moore et al. [20] and Teisner et al. [21]. These are based on first, purification of C3 from plasma, then the highly purified C3 and C3b are treated with commercial C3b inactivator or another proteolytic enzyme, followed by gel permeation on Sephadex G-200. However, these methods require large volumes of plasma (0.8–11 l) and expensive equipment.

The purpose of this work was to simplify the isolation procedure and reduce the purification cost of C3 complement component and C3d subunit of C3. This is achieved by using fraction IY-1 of Cohn's fractionation of human plasma as a raw material. According to the data of Cohn et al. [22] the precipitate IY-1, which is the waste of industrially fractionated human plasma from which albumin and immunoglobulins are extracted using cold ethanol, contains complement components [23]. We have determined that the IY-1 Cohn's fraction contains C3d and C3b fragments of the C3 complement component as a result of cleavage of C3 during the fractionation procedure.

2. Experimental

2.1. Materials

The fraction IY-1 of Cohn's fractionation of human plasma was obtained from the Factory of Blood Preparations (G.N. Gabrichevsky Research Institute of Epidemiology and Microbiology, Moscow, Russian Federation). The Diaflo ultrafiltration membranes were obtained from Amicon (Oosterhout, Netherlands). Rabbit anti serum specific to human C3 complement component was obtained from Behringwerke (Marburg, Germany) and rabbit antiserum specific to the C3d subunit of C3 complement component was obtained from DAKO Immunoglobulins (Copenhagen, Denmark). Rabbit anti-whole human serum protein was produced by us. Polyethylene glycol (PEG) 4000 was obtained from Loba Chemie (Vienna, Austria), EDTA potassium phosphate, sodium chloride, sodium azide, veronal, medinal and other chemicals were of analytical-reagent grade from Reachim (Moscow, Russian Federation); agarose was obtained from Sigma (St. Louis, MO, USA).

2.2. Sorbents

DEAE-Toyopearl 650 M was obtained from Toyo Soda (Tokyo Japan) and Sephacryl S 200 from Pharmacia (Uppsala, Sweden).

2.3. Immunological methods

Antigenic levels of the C3 complement component and C3d subunit of C3 in IY-1 Cohn's fraction and in purified fractions were evaluated by radial immunodiffusion according to Mancini et al. [24]. Immunoelectrophoresis in 1% agarose with veronal–medinal buffer (VMB) (pH

8.4–8.6), containing 0.02% sodium azide and 10 m*M* EDTA, was performed according to Scheidegger [25].

2.4. Isolation procedure

All procedures were performed at 4°C unless indicated otherwise. Centrifugation of PEG-precipitated solution was carried out at 6000 *g* for 20 min in a Janetzki K70D centrifuge.

Fractionation of the IY-1 Cohn's precipitate with PEG 4000 was carried out as follows. Approximately 20 g IY-1 Cohn's precipitate was dissolved in 100 ml of 0.1 *M* potassium phosphate buffer (pH 7.2–7.4) containing 10 m*M* EDTA and 0.5 *M* sodium chloride and the mixture was stirred for 24 h. Undissolved precipitate was removed by centrifugation. Then PEG 4000 was added to a 20% solution of IY-1 Cohn's precipitate with stirring to a final concentration of 16% PEG. After stirring for 30 min, the precipitate formed was separated by centrifugation. C3 and C3b were isolated from the 16% PEG precipitate and the C3d subunit was isolated from the 16% PEG supernatant.

2.5. Chromatography

Liquid chromatography

Liquid chromatography was carried out with a standard liquid chromatographic system (LKB, Bromma, Sweden), consisting of a Multiperpex pump, a Uvicord SII detector, a Superrac fraction collector and glass columns (18 × 2.1 cm I.D. and 50 × 2.1 cm I.D.) of our own construction. For protein detection the absorbance was measured at 280 nm with the LKB detector.

Ion-exchange chromatography

The 16% PEG precipitate, dissolved in 0.01 *M* potassium phosphate buffer (pH 7.4)–10 m*M* EDTA and the 16% PEG supernatant dialysed overnight against the same buffer were applied at a flow-rate 100 ml/h to a glass column (18 × 2.1 cm I.D.) of DEAE-Toyopearl 650 M equilibrated with 0.01 *M* potassium phosphate buffer (pH 7.4)–10 m*M* EDTA. Following application of the samples, the columns were washed with the same buffer at the same flow-rate until 300 ml of effluent had been collected and discarded. Then, C3 and C3b from the first column and C3d from the second column were eluted with a linear sodium chloride concentration gradient of 250 ml. Fractions of 6 ml were collected. C3- and C3d-containing fractions were pooled and concentrated by ultrafiltration on Amicon PM-30 and UM-10 membranes, respectively.

Gel permeation on Sephacryl S-200

Pools of selected proteins were applied to columns (50 × 2.1 cm I.D.) of Sephacryl S-200 equilibrated in 0.01 *M* potassium phosphate buffer (pH 7.5)–10 m*M* EDTA–0.15 *M* NaCl–0.02% NaN_3 at room temperature. Proteins were eluted at the flow-rate of 15 ml/h. Fractions of 5 ml were collected.

3. Results and discussion

The IY-1 precipitate of Cohn's fractionation of human plasma was used as a starting material for the purification procedure described here. It is the waste of industrially fractionated human plasma [22] and contains more than ten different proteins (α-1-antitrypsin, haptoglobin, ceruloplasmin, transferrin, haemopexin, α-1-lipoprotein, prealbumin, complement components, antithrombin III and others) in the ethanol precipitate [23]. We found that the IY-1 precipitate contains C3d and C3b fragments of C3 complement component, and used it for the isolation of these fragments. The ethanol concentration in IY-1 precipitate was 40%. The raw precipitate was pulverized and then dissolved in a buffer of high ionic strength such as 0.1 *M* potassium phosphate (pH 7.2–7.4)–10 m*M* EDTA–0.5 *M* NaCl for the dissociation of protein complexes. Undissolved precipitate was removed by centrifugation. Then about a 20% solution of IY-1 Cohn's fraction was precipitated with stirring for 30 min by adding 32% PEG 4000 solution to a final concentration of 16% PEG. The precipitate formed was separated by centrifugation.

The 16% PEG precipitate was found to con-

tain 42% C3 complement component compared with the initial C3 plasma, evaluated by radial immunodiffusion in addition to small amounts of the C3d fragment. Obviously, C3 complement component conversion occurs during plasma fractionation by cold ethanol. The normal concentration C3d fragment of the C3 complement component is about 3% of the initial plasma C3 [21]. Hence the normal concentration of C3d is about 0.036 g/l. The level of C3d in plasma increases because of acute or chronic activation of the complement system in vivo and activation of the complement system in vitro because of heating normal serum at 37°C, treatment with zymozan, $MgCl_2$, *E. coli* or aggregated IgG. The 16% PEG supernatant was found to contain 0.037 g/l of the C3d fragment of C3.

The next step was chromatography of the 16% PEG precipitate dissolved in 0.01 *M* potassium phosphate buffer (pH 7.4)–10 m*M* EDTA on the DEAE-Toyopearl 650 M column. C3 and C3b were eluted by a linear concentration gradient from 0 to 0.3 *M* NaCl. C3 and C3b were desorbed from the DEAE anion exchanger at 0.17–0.18 *M* NaCl (Fig. 1). C3-containing pool determined by immunoprecipitation was contaminated with α-globulins. A small amount of C3d dissociated from the DEAE anion exchanger at about 0.15 *M* NaCl. The 16% PEG supernatant dialysed against 0.01 *M* potassium phosphate buffer (pH 7.4)–10 m*M* EDTA was also chromatographed on the DEAE-Toyopearl 650 M column (Fig. 2).

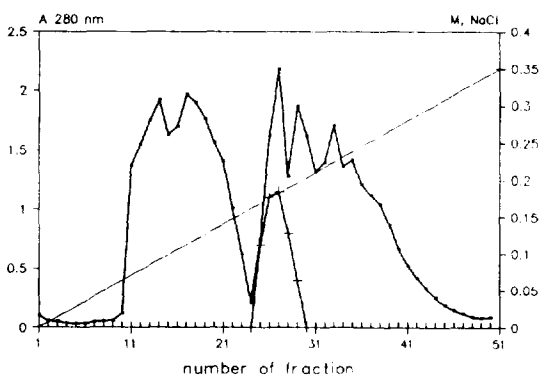

Fig. 1. Elution profile of the 16% PEG precipitate from the IY-1 Cohn's fraction on DEAE-Toyopearl 650 M. Elution conditions as described in the text. ■ = Absorbance at 280 nm; straight line = gradient of NaCl (from 0 to 0.35 *M*); + = C3 pool measured by radial immunodiffusion (Mancini et al's technique [24]) in 1% agarose gel prepared with 0.03 *M* veronal–medinal buffer (pH 8.6). The antiserum to the C3 complement component (Behringwerke) diluted 1:50 was added to the gel.

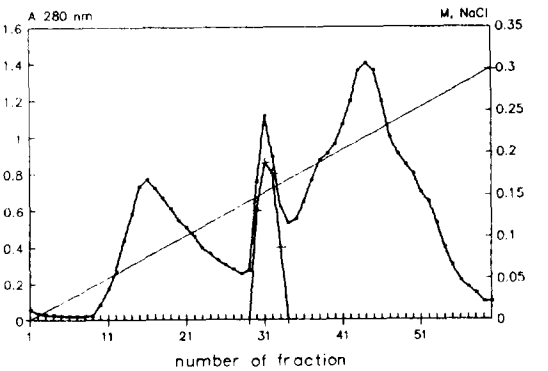

Fig. 2. Elution profile of the 16% PEG supernatant from the IY-1 Cohn's fraction on DEAE-Toyopearl 650 M. Elution conditions as described in the text. ■ = Absorbance at 280 nm; straight line = gradient of NaCl (from 0 to 0.30 *M*); + = C3d pool measured by radial immunodiffusion (Mancini et al.'s technique [24]) in 1% agarose gel prepared with 0.03 *M* veronal–medinal buffer (pH 8.6). The antiserum to the C3d subcomponent (DAKO) diluted 1:50 was added to the gel.

The next step was the concentration of pooled C3–C3b-containing fractions and C3d-containing

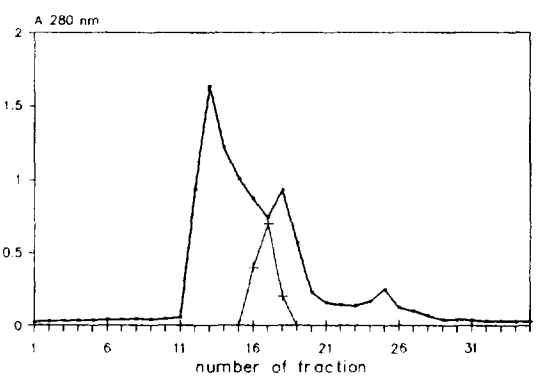

Fig. 3. Gel permeation of the post-DEAE C3–C3b pool on Sephacryl S 200. ■ = Absorbance at 280 nm; + = C3 complement component measured by Mancini et al.'s technique [24] as described in the caption to Fig. 1.

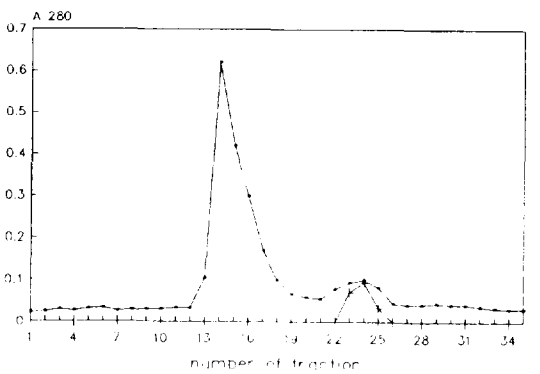

Fig. 4. Gel permeation of the post-DEAE C3d pool on Sephacryl S 200. ■ = Absorbance at 280 nm; + = C3d subcomponent of C3 complement component measured by Mancini et al.'s technique [24] as described in the caption to Fig. 2.

fractions by ultrafiltration on Amicon membranes. In the last step, concentrated pools of C3 + C3b and of C3d were purified on Sephacryl S 200 (Figs. 3 and 4). C3 obtained by the described method migrates in the β-2-region in immunoelectrophoresis. C3d obtained by the described method has a characteristic anodic mobility in immunoelectrophoresis described originally by West et al. [26] (Fig. 5).

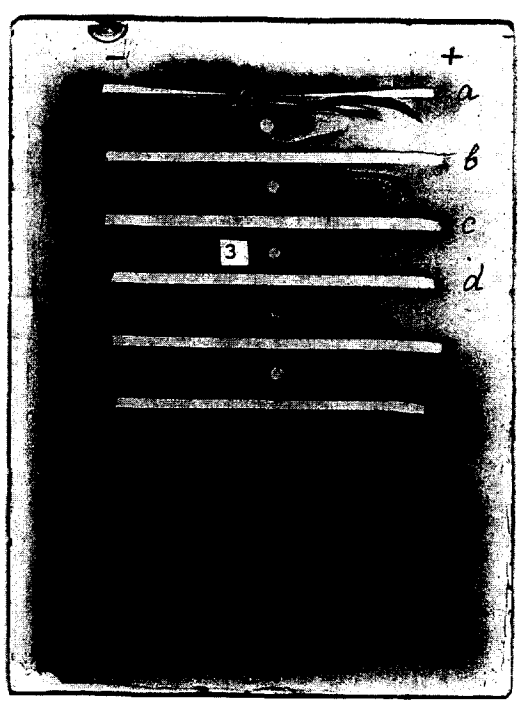

Fig. 5. Immunoelectrophoresis of the purified C3 complement component and C3d subunit of C3. Well: 1 = normal human serum; 2 = purified C3 + C3b; 3 = purified C3d. Troughs: (a) anti-whole human serum; (b) anti-C3 serum, (c, d) anti-C3d serum.

In conclusion, a simple method has been proposed for the isolation of C3 complement component and its C3d fragment from the IY-1 Cohn's precipitate using PEG 4000 as fractionating agent and two chromatographic steps, anion exchange and gel permeation.

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