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Isolation of Lipid-Free C-Reactive Protein by Affinity Chromatography

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Human C-reactive protein purification has been hampered by its association with lipids. Isolation of pure lipid-free C-reactive protein was obtained by a three step procedure. First, partially lipid-free C-reactive protein was obtained by affinity chromatography from ascitic fluids; second, lipid-bound proteins were eliminated by calcium-dependent precipitation; and third, lipid-free pure C-reactive protein was obtained by affinity re-chromatography of the supernatant. A 46-50 % yield of lipid-free C-reactive protein was obtained compared with the 14.7 % obtained by the old method of extraction with lipid solvents.

Key words: C-Reactive protein, Lipid-free, Purification.

C-reactive protein (CRP) is a human plasma protein rising in concentration with tissue injury, infection or inflammation, and returning to normal levels as recovery occurs (4). The CRP molecule consists of 5 identical polypeptide monomers (molecular weight of about 21,000 each) noncovalently associated in a disc-like configuration with cyclic pentameric simmetry. CRP undergoes calcium-dependent binding to phosphorylamine compounds such as phosphorylcholine, and phosphorylethanolamine (3, 7). Preparation of large amounts of pure CRP has been hampered by its association with lipids or lipoproteins (9, 10, 12). WOOD *et al* (12) described a method for preparation of lipid-free CRP that still is in use today, which consisted in stirring a citrate solution (calcium-free) of lipid-bound CRP over chloroform. SATO an HARA (11) used lyophilization followed by chloroform-methanol extraction. Both of these methods produced extensive denaturation of CRP and therefore low yields of lipid-free CRP.

The present paper describes a simple method to dissociate lipid from CRP and recover lipid-free CRP in higher yields.

Materials and Methods

Isolation of CRP.— It was isolated from ascitic fluids removed for therapeutic purposes from patients with disseminated mammary carcinomas. The fluids were clarified by filtration through Whatman

no. 3 MM paper. They were stored at −20° C until use. Two methods were used to purify human CRP, one based on ammonium sulfate precipitation and chloroform extraction (12), and the other one based on affinity chromatography (9, 10), modified as follows: for the affinity chromatography method, either 700 ml of human ascitic fluids or partially lipid-free CRP were dyalized against 20 mM Tris, 0.1 M NaCl, 10 mM $CaCl_2$, pH 8 (calcium-containing buffer) and then passed through a column of Sepharose 4 B, 8 × 3 cm (Pharmacia, Uppsala, Sweden), connected to another column of 7 × 1.2 cm of agarose immobilized 2-aminoethyl-dihydrogen phosphate (Pierce Chemicals, Rockford, Ill., USA). After washing the second column with 10 volumes of the dialysis buffer described above, it was eluted with 20 mM Tris, 0.1 M NaCl, 20 mM citrate, pH 8 (calcium-free buffer). The Sepharose 4 B column binds the serum amyloid P component (a major contaminant in the isolation of CRP). The 2-aminoethyl-dihydrogen phosphate column binds CRP. CRP pure standard was obtained from Sigma.

Identification of CRP.— It was made by immunodiffusion against commercial anti-CRP antiserum, polyacrylamide gel electrophoresis, blotting and by enzyme-linked immunoassay.

Immunodiffusion was in 200 mM borate, 75 mM NaCl, 2.5 mM $CaCl_2$, 1 % agar, 100 mg/l merthiolate, pH 8 against anti-CRP antiserum (Biomerieux, France). A 15 % polyacrylamide gel electrophoresis in 0.1 % SDS was used in determining purity (5). The total protein content of crude CRP was measured by A280 nm, assuming $\varepsilon^{1\,\%}_{1\,cm} = 19.5$ (8). For the blotting experiments, CRP (5 µg/well) was separated by 15-20 % polyacrylamide gel electrophoresis without SDS, in 100 × 80 × 1 mm slab gels. Once the gel was run it was transferred to nitrocellulose paper by electrotransfer in 3 g Tris, 28.8 g glycine/l, pH 8, using an apparatus from Biorad at 60 V for 1 hour. After transfer, the nitrocellulose paper was incubated with 20 mM phosphate, 150 mM NaCl, pH 7.4, 1 % BSA, 0.5 % milk during 30 min at 37° C for blocking. Then it was incubated with anti-CRP antisera conjugated to peroxidase (2), diluted in the same buffer with 0.05 % Tween 20 during 30 min at 37° C and afterwards washed thoroughly with 20 mM Tris, 150 mM NaCl, pH 7.4. The bands were stained by immersion of the nitrocellulose paper in 10 ml of 20 mM Tris, 150 mM NaCl, pH 7.4 with 3 mg/ml of 4-chloro-naphtol in 2 ml of methanol and 1 % H_2O_2. CRP concentration was measured by CRP binding to solid-phase plates coated with phosphorylethanolamine (PE) as described (6).

Antisera against partially lipid-free CRP.— To obtain antibodies against partially lipid-free CRP, goats and rabbits were used. Two goats (30 kg weight) were intramuscularly injected daily with a total of 1 mg of partially purified CRP (fraction II, fig. 1 A) in 5 ml of 50 mM sodium phosphate, pH 7.4 mixed with 5 ml of Freund's adjuvant. Five rabbits (5 kg of weight) were injected daily with a total 100 µg of the same fraction of CRP in 1.2 ml of 50 mM sodium phosphate, pH 7.4 with 1.2 ml of Freund's adjuvant (Difco, Detroit, Mich., USA). Complete Freund's was used in the first injections; 5 more were given with incomplete Freund's. Sixty days after the last injection, animals were bled by the jugular (goats) or by the ear vein (rabbits). Blood was allowed to clot and serum was obtained by centrifugation and frozen until use.

Isolation and analysis of lipids.— The CRP fractions were added to 15-20 ml of chloroform-methanol (1:2) and lipids were extracted by the method of BLIGH and DYER (1). The lipid extract was con-

centrated using a rotaryevaporator and stored at −20° C in atmosphere of nitrogen. The lipid residue was dissolved in chloroform-methanol (1:2) and aliquots were fractionated by thin-layer chromatography over silicagel G (Merck) plates of 500 μ thick. Hexane-ether-acetic acid: 70:30:1 (v/v/v) was used to separate neutral lipids and chlorophorm-methanol-distilled water 65:25:4 (v/v/v) was used to separate polar lipids. The lipid spots were visualized with iodine, marked with a needle and identified by means of known standards.

Results

Figure 1 A shows the results of a typical affinity chromatography of human ascitic fluid over agarose immobilized 2-aminoethyl-di-hydrogen phosphate. Peaks I and II were obtained. Human ascitic fluid measured by CRP enzyme-linked immunoassay (detection limit 0.34 ng/well), contained 60 mg of CRP/l. After passing 700 ml of human ascitic fluid by the affinity chromatography columns, the amount of CRP was non-detectable by the enzyme-linked immunoassay.

Washing with the calcium-containing buffer (more than 10 column volumes, overnight elution or even several days incubation in calcium-containing buffer) did not reduce CRP or lipid-bound to the column. Only the use of calcium-free buffer allowed the elution of both CRP and lipids (fig. 1 A). Five human ascitic fluids were fractionated as indicated above. Percentage of peak I with respect to peak II varied from 10 to 125 % (average 43.4 %). The amount of peak II varied from 40 to 190 μg per ml of starting ascitic fluid (average 80 μg/ml).

Pooled peak I (fig. 1 A) was opalescent and showed an abundant precipitate after storage at 4° C. Peak I did not give any precipitation bands by immunodiffusion against anti-CRP commercial antisera.

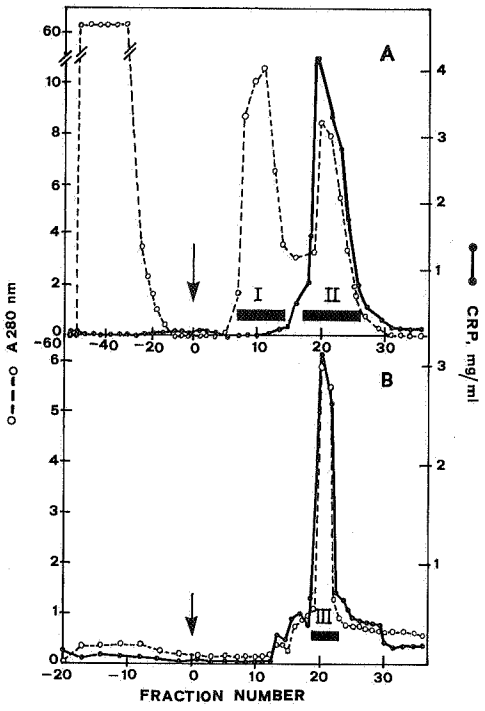

Fig. 1. *Affinity purification of CRP from human ascitic fluids (A) and re-purification of peak II (B).* Seven hundred ml of human ascitic fluids dialyzed against 20 mM Tris, 0.1 M NaCl, 10 mM $CaCl_2$, pH 8 were filtered and passed through a column of agarose immobilized 2-aminoethyl-di-hydrogen phosphate (7 × 1.2 cm). After washing the column with the dialysis buffer, the column was eluted with 20 mM Tris, 0.1 M NaCl, 20 mM citrate, pH 8 (the arrow indicates the begining of elution). Fractions with CRP activity (peak II) were pooled and the process was repeated again (B), discarding the precipitated material after dialysis. Fractions were 1 ml. Black horizontal bars indicate the fractions pooled. o - - - o A 280 nm, ● - - - ● mg/ml of CRP as measured by enzyne immunoassay (6).

CRP content was not detectable by enzyme immunoassay. Peak I showed several protein bands by denaturing PAGE stained with Coomassie blue (fig. 2) but only when concentrated 10-fold.

Pooled peak II (fig. 1 A) was opalescent and showed a precipitate after storage at 4° C. Peak II gave a strong precipitation band by immunodiffusion against anti-CRP commercial antisera. CRP concentration of peak II varied from 1 to 10 mg/ml as measured by enzyme immunoassay. Peak II showed 2 major protein bands at 21 Kd and on top of the gel and some minor protein bands in the 70-90 Kd region by denaturing PAGE stained with Coomassie blue. The band at 21 Kd corresponded to pure CRP as demonstrated by pure commercial CRP run in parallel (fig. 2) and by reaction with peroxidase labelled anti-CRP antisera by blotting.

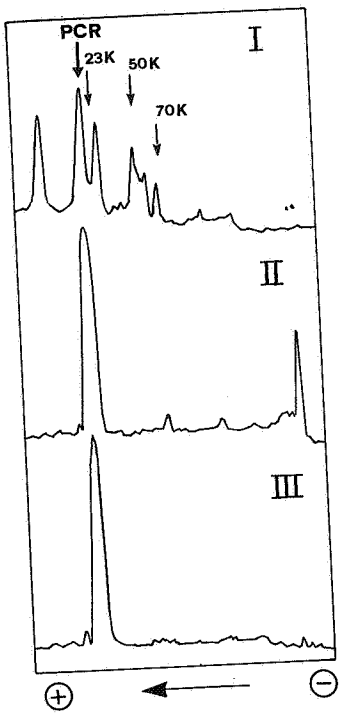

Fig. 2. *Comparison of peaks I, II and III by SDS-polyacrylamide gel electrophoresis.* Electrophoresis was in 15 % polyacrylamide slabs containing 0.1 % SDS. Samples were heated to 90 °C 2 min in sample buffer containing 5 % 2-mercaptoethanol and 1 % SDS. Goat IgG (50 KDa and 23 KDa) and trout IgM (70 KDa and 23 KDa) immunoglobulins were used as molecular weight markers. Gels were stained by Coomassie, dried and scanned with a densitometer Chromoscan 3 Joyce Loebl (Gateshead, England). Peak I was concentrated 10 fold relatively to peaks II and III.

Dialysis of peak II against the calcium-containing buffer precipitated 35 to 85 % of the absorbance at 280 nm. After removing the precipitate by centrifugation, peak II was passed through the two columns again (fig. 1 B). Peak III was obtained after eluting the column with calcium-free buffer. The yield of peak III was 46-50 % of the absorbance at 280 nm applied to the column (fig. 1 B). Material pooled from peak III was clear, showing no precipitate after storage for more than one year at 4° C, one precipitation line by immunodiffusion against anti-CRP commercial antisera and only one stained band at about 21 Kd by denaturing PAGE stained with Coomassie blue (fig. 2). No precipitate formed when peak III was dialyzed overnight against calcium-containing buffer. Moreover, protein content (measured by A280) and CRP activity (measured by CRP binding to solid-phase phosphorylethanolamine) agreed 88 ± 9 percent (average ± S.D., n = 2).

Thin-layer chromatography of lipids extracted from peaks I and II (fig. 1 A) showed a similar lipid composition of both peaks. Main composition was cholesterol, esters of cholesterol and triglycerides (fig. 3 A). Very low amounts of polar lipids were detected (fig. 3 B). No lipids were found associated with peak III (data not shown).

Peak III, II and I were analyzed by double immunodiffusion against anti-peak II antisera from either rabbits or goats. Peak III gave only one line of precipitation with anti-peak II antisera. Peak II gave several lines of precipitation with anti-peak II antisera, one of which was of total identity with the peak III-anti-peak II precipitation line. Peak I also gave sev-

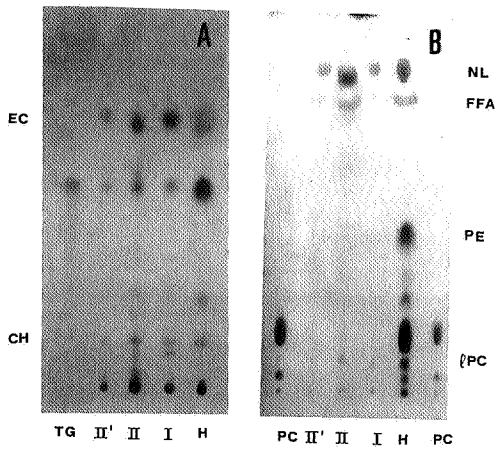

Fig. 3. *Thin-layer chromatography of lipids associated to CRP.*
Hexane-ether-acetic acid: 70:30:1 (v/v/v) was used to separate neutral lipids (A) and Chloroform-methanol-water: 65:25:4 (v/v/v) was used to separate polar lipids (B). PC: phosphatidylcholine, PE: phosphatidylethanolamine, LPC: lisophosphatidylcholine, NL: neutral lipids, FFA: free fatty acids, TG: triglycerides, CH: cholesterol, EC: esters of cholesterol, H: lipid extract from mouse liver, I: peak I from figure 1 A, II: peak II from figure 1 B (II is about 5 fold more concentrated than II'). Lipids isolated from 2 different ascitic fluids showed similar composition.

eral lines of precipitation with anti-peak II antisera, some of them were of total identity with peak II-anti-peak II precipitation lines but none was of identity with peak III-anti-peak II precipitation line.

Discussion

A CRP precipitate dependent on the presence both of calcium and lipids was found by MAC LEOD and AVERY during their earlier isolation attemps (4). Defatted serum no longer yielded fractions that precipitated on calcium addition (12). This fact was used as a criterion for purity of lipid-free CRP, but only 14.7 % of intact CRP could be recovered by this method (data not shown). In the present studies, and in the presence of calcium, lipid-bound CRP from human ascitic fluids could be isolated by binding to agarose immobilized 2-aminoethyl-di-hydrogen phosphate columns (fig. 1 A). When calcium is omitted, some lipids were eluted first (peak I) and some lipids were eluted together with CRP (peak II). Upon dialysis of peak II in the presence of calcium, the lipids reassociated with CRP to precipitate but 46-50 % of the CRP remained free from lipids and therefore it could be isolated pure (peak III) by rechromatography (fig. 1 B).

The presence of CRP was demonstrated by double immunodiffusion against commercial antisera to human CRP, and its purity was assessed by polyacrylamide gel electrophoresis (fig. 2), and blotting. Furthermore the CRP values of 99 human sera as determined by commercial nephelometry (x) and by enzyme-linked (y) immunoassay (using CRP binding to solid-phase phosphorylethanolamine and standards made with CRP purified by the method described in this paper) gave correlation values of $r = 0.96$ with a regression equation in mg/l of $y = 1.3x + 3$ (10). On the other hand the analysis of the lipids associated to CRP shows (fig. 3) cholesterol ester, cholesterol and other neutral lipids confirming the results obtained by SATO and HARA (11). All the above mentioned data show the high purity of the lipid-free CRP isolated by means of the method described here.

The presence of other proteins in peak II could allow lipids to be in association with those proteins rather than with CRP. Furthermore, some common non-CRP proteins are present in peak I and peak II as shown by immunodiffusion experiments using anti-peak II antibodies. If this were the case, the calcium-dependent association of CRP and lipid could be through a protein to protein interaction.

Resumen

Se presenta una técnica de aislamiento de la proteína C reactiva libre de lípidos, por un procedimiento en tres pasos: 1) purificación parcial por cromatografía de afinidad, a partir de fluidos ascíticos; 2) eliminación de las proteínas ligadas a lípidos, mediante precipitación en presencia de calcio; y, 3) obtención de la proteína C reactiva purificada y libre de lípidos, por recromatografía del sobrenadante. Por este procedimiento se obtiene un rendimiento del 46-50 %, mientras que por el método convencional, por extracción con solventes, se obtiene un 14,7 %.

Palabras clave: Proteína C reactiva, Purificación, Sin lípidos.

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