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Isolation of C-reactive protein by affinity chromatography

M. B. PEPYS, A. C. DASH & M. J. ASHLEY *Department of Immunology, Royal Free Hospital, Pond Street, London*

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SUMMARY

Different affinity chromatography methods were compared for the isolation of C-reactive protein (CRP) from pathological fluids. In the presence of calcium ions CRP became bound to agar (Ganrot & Kindmark, 1969), to cyanogen bromide-activated agarose beads (Sepharose 4B) coupled with pneumococcal C substance (Osmand *et al.*, 1975), to glutaraldehyde-activated polyacrylamide beads (Bio-Gel P300) or agarose–acrylamide beads (Ultrogel AcA 34) coupled with C substance and to 'sulphated' polyacrylamide (Bio-Gel P300) beads. After thorough washing, elution with citrate or EDTA yielded CRP of varying degrees of purity. The use of agar provided high yields of heavily contaminated CRP which was substantially purified by passage through a column of insolubilized anti-normal human serum antibodies. Insolubilized C substance provided much purer CRP and the best yields were obtained from Sepharose–CNBr–C substance to which was attached considerably more ligand than was coupled to glutaraldehyde-activated beads. The only significant contaminant of the CRP isolated in this way was a normal serum protein, the P component of amyloid (C1t), which showed calcium-dependent binding to plain unsubstituted Sepharose and Ultrogel, as well as to polyacrylamide beads which had been treated with concentrated sulphuric acid. The use of these easily prepared 'sulphated' polyacrylamide beads provides a method for isolating small quantities of CRP contaminated only by P component (C1t), without the need to first prepare C substance. Separation of CRP from P component was readily achieved by absorption of the P component onto agarose beads in the presence of calcium ions.

An 'electroimmunodiffusion', 'rocket' type of assay for pneumococcal C substance was developed, using CRP-rich fluid in the gel instead of antiserum.

INTRODUCTION

Osmand *et al.* (1975) have isolated CRP from pathological fluids by calcium-dependent affinity chromatography using a solid-phase absorbent of pneumococcal C substance covalently linked to Bio-Gel A (agarose) beads. In view of the capacity of CRP to bind in the presence of calcium ions to other ligands, we have investigated different affinity chromatography methods of purifying CRP and compared them with the use of C substance. In the course of these studies we also isolated, as a result of its calcium-dependent binding, another serum protein, which unlike CRP was present in comparable amounts in normal and pathological samples. This was later identified (Pepys *et al.*, 1977b) as the plasma or P component of amyloid (Cathcart, Comerford & Cohen, 1965; Skinner *et al.*, 1974). An apparently novel serum protein isolated in the course of studies on C1, and called C1t (Assimeh & Painter, 1975), has since been found to be identical with P component (Painter *et al.*, 1976). We refer here to this protein as P component.

MATERIALS AND METHODS

CRP-rich fluids. Pleural and ascitic fluids aspirated for diagnostic or therapeutic purposes were clarified by centrifugation and filtration, and stored at 4°C after addition of sodium azide to 0·1%w/v.

Correspondence: Dr M. B. Pepys, Department of Medicine, Royal Postgraduate Medical School, Du Cane Road, London W12 0HS.

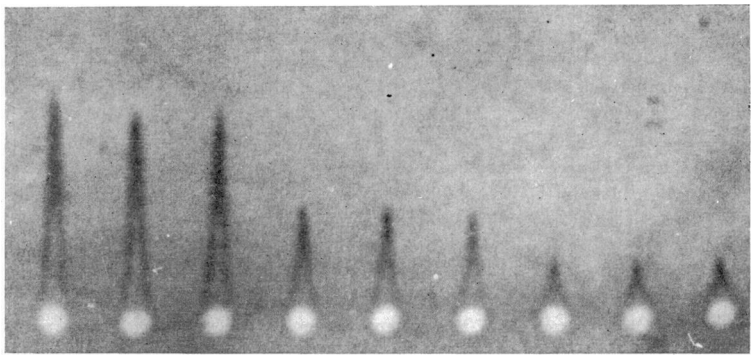

FIG. 1. Assay of pneumococcal C substance by electrophoresis in agarose gel containing CRP-rich fluid. Three replicates are shown of different standard concentrations.

Estimation of CRP and P component (*C1t*). The concentrations of CRP and P component in fluids and column eluates were measured by electroimmunodiffusion (Laurell, 1966; Kindmark, 1969), using a rabbit antiserum which contained antibodies against both proteins (see below). Standard samples of the isolated purified proteins were used for calibration in terms of μg protein/ml.

Estimation of pneumococcal C substance. The concentration of pneumococcal C substance was measured, in order to assess efficiency of coupling to chromatography materials, by an electroimmunodiffusion type of assay. Pathological fluid containing 80 μg CRP/ml was incorporated at a final concentration of 20% v/v in 1% agarose gel in 0·075 M veronal buffer, pH 8·6, containing 2 mM calcium chloride. Samples to be assayed for C substance were placed in wells in this gel and subjected to electrophoresis at 10 V/cm using the same veronal buffer in the troughs. 'Rockets' of precipitation were formed which, after washing and pressing the gel, were stained with Coomassie blue or Amido black. The assay was calibrated with standards prepared from isolated C substance (Figs 1 & 2).

Chromatography media. 'Ionagar' No. 2 (Oxoid Ltd, London S.E.1) was used to prepare a 5% agar gel in CFT diluent (Oxoid Ltd) which was homogenized in a Waring blender before use. Bio-Gel P beads (100–200 mesh) were obtained from Bio-Rad Laboratories Ltd, Kent; Ultrogel AcA 34 beads from LKB Producter AB, S 161 25 Bromma 1, Sweden; Sepharose 4B beads and Sepharose 4B–CNBr were obtained from Pharmacia (GB) Ltd, London W5.

Pneumococcal C substance. Pneumococcal C substance was isolated from cultures of a Cs capsulated pneumococcal variant (kindly provided by Dr Gerald Schiffman, Department of Microbiology, Downstate Medical Centre, Brooklyn, N.Y., U.S.A.) as described by Gotschlich & Liu (1967), except that enzymatic digestion was omitted.

Other ligands. O-phosphoethanolamine and choline phosphate chloride were obtained from Sigma London Chemical Co. Ltd, Surrey; protamine sulphate from Boots Ltd, Nottingham and concentrated sulphuric acid from BDH Chemicals Ltd, Dorset.

Coupling procedures. Coupling of various ligands to Bio-Gel P300 (100–200 mesh) polyacrylamide beads and Ultrogel AcA 34 acrylamide–agarose beads was attempted by the method of Ternynck & Avrameas (1976) using glutaraldehyde (EMscope Ltd, London). After coupling, excess reactive groups were blocked with ethanolamine. Sepharose 4B was acti-

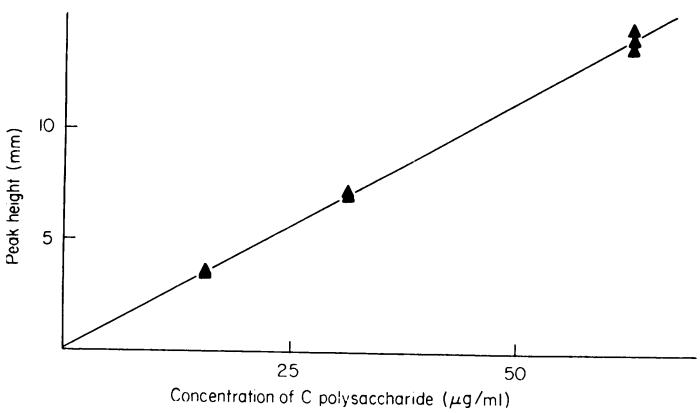

FIG. 2. Calibration curve for the 'rocket' assay of pneumococcal C substance using CRP-rich fluid, illustrating the rectilinear relationship between concentration and peak height.

vated with cyanogen bromide (Sigma London Chemical Co. Ltd, Surrey), using 300 mg/ml beads at 0°C and pH 11·0. 10–20 mg of IgG isolated by salt precipitation from polyvalent anti-normal human serum were coupled per ml activated beads at pH 7·0 in 0·5 M NaCl–0·01 M phosphate buffer, and excess reactive groups then blocked with 1·0 M ethanolamine, pH 8·0. Pneumococcal C substance was offered for coupling to commercially available Sepharose 4B–CNBr beads according to the manufacturer's instructions. (Pharmacia (GB) Ltd, London W.5).

'Sulphation' of Bio-Gel P beads. Bio-Gel P300 beads were swollen in distilled water; equal volumes of settled beads and undiluted concentrated sulphuric acid were mixed at room temperature and then left for 10 min before washing with distilled water. Washing was continued until no further free sulphate was detectable by precipitation with barium chloride.

Isolation of CRP by calcium-dependent affinity chromatography. Solid-phase ligands were equilibrated with Tris-buffered isotonic saline containing 1 mM calcium before being allowed to react with CRP-rich fluids, either as a batch procedure (agar, Bio-Gel P300 beads) or in a column (Ultrogel, Sepharose), for at least 1 hr at room temperature, and often overnight at 4°C thereafter. Sufficient CRP-rich fluid was provided in each experiment to saturate the capacity for CRP of the affinity medium, which was then washed extensively with Tris–saline–calcium until the washings (or column effluent) was free of material absorbing at 280 nm. CRP was then eluted with either Tris-buffered saline, pH 8·0, containing 0·05 M sodium citrate or with phosphate-buffered saline, pH 7·3, containing 0·01 M EDTA (PBS–EDTA). The concentrations of CRP in the starting fluid, the absorbed fluid and the eluted material were measured in each experiment, and the purity of the eluted CRP assessed by gel diffusion and electroimmunodiffusion testing against polyvalent sheep anti-normal human serum.

RESULTS

Isolation of CRP and P component using agar and raising of antiserum

The initial preparation of CRP utilized the adsorption of CRP onto agar in the presence of calcium ions, as described by Ganrot & Kindmark (1969). After thorough washing, the CRP was eluted with PBS–EDTA, but was heavily contaminated with other serum proteins. It was substantially purified by passage through a Sepharose 4B column bearing anti-normal human serum antibodies linked via cyanogen bromide, but still contained a single contaminant which reacted in gel diffusion with an antibody present in low titre in the anti-normal human serum. Addition of 20% w/v sodium sulphate apparently precipitated this impurity and the resulting CRP preparation, which reacted strongly with a commercial goat anti-human CRP, no longer reacted with anti-normal human serum.

A rabbit was immunized by injection of 5 mg of this CRP in Freund's complete adjuvant into multiple subcutaneous sites, and it was subsequently boosted by injection of a further two lots of 2 mg of the same preparation in incomplete adjuvant. The antiserum which resulted reacted strongly with CRP and also, more weakly, with a normal serum protein which was later identified as P component (Pepys *et al.*, 1977b).

Attempted isolation of CRP using ligands coupled to glutaraldehyde-activated Bio-Gel P300 beads

Attempts were made under a variety of conditions to couple phosphoethanolamine, choline phosphate and protamine sulphate (CRP is known to react with all of them) to glutaraldehyde-activated Bio-Gel P300 beads. The coupled beads were used to absorb CRP from pathological fluids, which was then eluted off by chelation of calcium ions. Small yields of CRP were obtained from the choline phosphate and phosphoethanolamine beads, but other proteins were always present and the method therefore had no practical value. Beads to which coupling of protamine sulphate was attempted yielded no CRP.

Isolation of CRP using 'sulphated' polyacrylamide beads

Bio-Gel P beads which had been exposed to concentrated sulphuric acid and then washed until no more free sulphate eluted, were able to bind significant amounts of CRP, up to 180 μg/ml of beads in the case of P300. After extensive washing, elution of the beads with Tris–citrate–saline or PBS–EDTA yielded up to 40 μg CRP per ml of P300 beads. This CRP precipitated with C substance and contained only a single immunochemically detectable contaminant, which was P component.

Although the 'sulphated' P300 beads were the most efficient at binding and then yielding CRP, all the other Bio-Gel P beads tested except P2 behaved similarly. Furthermore, all the sulphated beads also bound and yielded P component. Untreated Bio-Gel P beads adsorbed neither CRP nor P component.

TABLE 1. Isolation of CRP by affinity chromatography on insolubilized pneumococcal C polysaccharide

Matrix	Coupling agent	Binding capacity*		Yield*	
		CRP	P component	CRP	P component
Bio-Gel P300†	Glutaraldehyde	456	51	266	4
Ultrogel AcA 34‡	Glutaraldehyde	1178	214	636	103
Sepharose 4B§	Cyanogen bromide	1617	n.d.	1198	10

n.d., Not done.

* Results shown are μg protein/mg coupled C substance, representative of four individual experiments.

† 10 ml beads bearing 3·0 mg C substance.

‡ 10 ml beads bearing 3·3 mg C substance.

§ 7 ml beads bearing 16·7 mg C substance.

Isolation of CRP using insolubilized pneumococcal C polysaccharide

C substance was coupled to glutaraldehyde-activated Bio-Gel P300 and Ultogel AcA 34 beads, and also to cyanogen bromide-activated Sepharose 4B. The relative capacities for, and yields of, CRP of these different materials are shown in Table 1. The greater efficiency of the Sepharose–CNBr–C substance column may reflect the fact that it bore much more C substance per ml of beads than the other two supports. In all cases the eluted CRP was contaminated with P component, although this was proportionately less in the case of the CRP eluted from the Sepharose–C substance column and was minimal in the CRP eluted from P300–C substance beads (Table 1). Glutaraldehyde-activated Bio-Gel P300 beads which were blocked with ethanolamine, without coupling of any ligand, failed to bind either CRP or P component.

Calcium-dependent binding of P component to agarose

In order to assess the capacity of unactivated, unsubstituted agarose to bind CRP, pathological fluids were passed through columns of plain Sepharose 4B or Ultrogel AcA 34. After extensive washing with Tris–calcium–saline, elution with Tris–citrate–saline or PBS–EDTA yielded only trace amounts of CRP. However, substantial quantities of P component were first bound and then eluted, indicating that this protein has a calcium-dependent affinity for agarose.

Further purification of CRP

The only significant contaminant of CRP isolated by calcium-dependent affinity chromatography on 'sulphated' Bio-Gel beads or C substance coupled to polyacrylamide, agarose or acrylamide–agarose beads was P component. This could be removed by gel filtration in Tris–citrate–saline, taking advantage of the appreciable difference of molecular weight between CRP (123,000) and P component (225,000). Gel filtration also separated CRP from its aggregates and subunits (Osmand *et al.*, 1975). Alternatively, absorption of the CRP containing P component with Sepharose or Ultrogel beads in the presence of calcium ions removed all the P component as a result of its binding to agarose. The optimal procedure was therefore to undertake gel filtration of the CRPplus P component mixture on Ultrogel AcA 44 beads in Tris–calcium–saline. This combined molecular seive purification of the CRP with affinity chromatography removal of the P component.

DISCUSSION

Ordinary agar gel has a high capacity for binding CRP, but the material which is eluted by citrate or EDTA contains many other proteins. These can mostly be removed by passage over a column of

insolubilized antibodies to normal serum proteins. The exception, at least in the case of the antiserum we used, was P component, against which there was insufficient antibody present. Inclusion of a high titre of antibodies against this antigen, or alternatively elution of the Sepharose–anti-normal human serum column with a calcium-containing buffer (see below) should enable this preparative method to yield substantial quantities of purified CRP.

Our failure to completely remove contaminating P component from the CRP preparation which we used to raise anti-CRP, despite precipitation of the material with sodium sulphate, fortunately led to the production of a combined anti-CRP plus anti-P component serum. The use of this antiserum to monitor isolation of CRP enabled us to detect concurrent separation of P component. We demonstrate here for the first time that this substance, which is a normal serum protein, undergoes calcium-dependent binding to agar, agarose and sulphated polyacrylamide. Its presence is therefore inevitable in citrate or EDTA eluates of these media when they have been exposed to sera or exudate fluids in the presence of calcium ions. However, use of only polyvalent anti-normal human serum, which might contain little, or even no, antibody to P component, might fail to reveal traces of P component in an apparently pure CRP preparation. Further studies of the isolation and characteristics of P component are reported elsewhere (Pepys *et al.*, 1977a, b).

Attempts to prepare affinity chromatography media for the isolation of CRP by coupling of phosphoethanolamine, choline phosphate or protamine sulphate to glutaraldehyde-activated Bio-Gel P300 beads were unsuccessful. The little CRP which bound to, and then eluted from, such beads was heavily contaminated with other serum proteins.

In marked contrast, Bio-Gel P300 beads, which had been briefly exposed to sulphuric acid, were able to bind and yield appreciable quantities of CRP, the only contaminant of which was P component which underwent a calcium-dependent interaction with the beads similar to that of CRP. The separation of P component from CRP is straightforward, as discussed below, so that the use of 'sulphated' polyacrylamide provides a simple affinity method for isolating CRP without first having to prepare pneumococcal C substance. The main disadvantage of the method is that the yield of CRP per ml of beads is low and tends to decrease with repeated use of the same beads.

The isolation of CRP using pneumococcal C substance coupled to cyanogen bromide-activated agarose beads was described by Osmand *et al.* (1975). We have confirmed that the method is very efficient, readily providing large yields of substantially purified CRP, but this was nonetheless contaminated by some P component. The contamination results from the use of agarose as the matrix. When glutaraldehyde-activated Bio-Gel P300 beads bearing C substance were used, very little P component was present; whilst Ultrogel (acrylamide–agarose)–glutaraldehyde–C substance beads yielded appreciable P component. The ratio of P component to CRP in the citrate eluates depends on the relative quantities of agarose and C substance.

Glutaraldehyde-activated Bio-Gel P300 and Ultrogel AcA34 coupled relatively less C substance per ml of beads than did cyanogen bromide-activated Sepharose. A small column of Sepharose bearing a lot of C substance was therefore able to yield substantial quantities of CRP containing only traces of P component, and it is therefore concluded that this is the matrix of choice for isolation of CRP.

Separation of CRP from P component was most conveniently achieved by taking advantage of the calcium-dependent binding of the latter to agarose, whilst CRP associates only very weakly with agarose. Combination of this affinity purification with gel filtration on Ultrogel AcA 44 permitted the separation in one step of CRP from its aggregates, subunits and other possible impurities.

Finally, we have devised a simple and sensitive assay for pneumococcal C substance using CRP-rich fluid instead of antiserum in an electroimmunodiffusion ('rocket') type of assay. This technique facilitates measurement of the efficiency of coupling of C substance to activated beads, and should be useful in other studies of C substance.

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