ONE STEP PREPARATION OF BOTH HUMAN C-REACTIVE PROTEIN AND CIt

M. PONTET, R. ENGLER and M. F. JAYLE

UER Biomédicale des Sts-Pères, Service de Biochimie, Laboratoire Associé n° 87 du CNRS, 45, rue des Sts-Pères, 75270 Paris Cédex 06, France

Received 14 December 1977

1. Introduction

C-reactive protein (CRP) is a trace constituent of normal human sera, which increases as much as 1000-fold in many inflammatory reactions [1]. Comparison of amino acid sequences of many different proteins has shown the closest relationship between CRP and CIt. Moreover, examination of these proteins by negative stain electron microscopy revealed a similar cyclic structure of five subunits. The term pentraxin has been therefore proposed to describe these related proteins [2]. These data have been confirmed by complete amino acid sequence of human CRP [3]. Otherwise CIt appears to be identical to serum P-component and to 9.5 S α_1-glycoprotein [4,5].

CRP preparation using affinity for the pneumococcal C-polysaccharide, which is at the origin of the discovery and the name of CRP [6], is still widely employed, particularly for sequence determinations and biological interactions studies. This practice is maintained, although the preparation of pneumococcal C-polysaccharide is unanimously recognized as tedious. In fact, the other proposed methods are not any more convenient [7–10].

This paper describes a fast method to prepare CRP by affinity chromatography with a high yield. This preparation takes advantage of the calcium dependent affinity of CRP for numerous ligands. The chosen system allows successive elution of CRP and CIt, both obtained as pure constituents, according to immunological and electrophoretic criteria.

2. Materials and methods

Sepharose 4B and activated CH Sepharose 4B were purchased from Pharmacia Fine Chemicals Co., Uppsala. Immunoelectrophoresis was performed on 1.5% agarose in 0.05 M barbital buffer, pH 8.6, by using antiserum to whole human serum (Hyland-Travenol Lab. Inc. Costa Mesa, CA), or monospecific anti-CRP and anti-CIt sera, obtained from ICl Scientific, Fountain Valley, CA and from Atlantic Antibodies, Westbrook, ME. CRP determinations were performed by single radial immunodiffusion (LC Partigen plate, Behringwerke AG, Marburg, FRG). Polyacrylamide gel electrophoresis was carried out in 7.5% acrylamide and gels were stained with Coomassie brilliant blue. The affinity chromatography column was prepared by the following method: 10 g freeze-dried activated CH Sepharose 4B were suspended in 10^{-3} M HCl, and washed on a sintered-glass filter with 2 l 10^{-3} M HCl. One gram of ligand 2-amino-ethanol dihydrogenphosphate (Merck-Schuchardt), was dissolved in 25 mM borate buffer, 0.5 M NaCl, pH 7.8. Coupling was performed by gently mixing the suspension during 2 h, at 30°C. The gel was left in contact 1 h with 1 M ethanolamine, in borate buffer, in order to block any remaining active group. The ethanolamine was removed by filtration, and the gel washed 3 times alternately, with 0.1 M acetate buffer, 1 M NaCl, pH 4, and with 0.5 M Tris–HCl buffer, 0.5 M NaCl, pH 8. The gel (30 ml) was packed in a glass column (15 × 200 mm). Elu-

Elsevier/North-Holland Biomedical Press

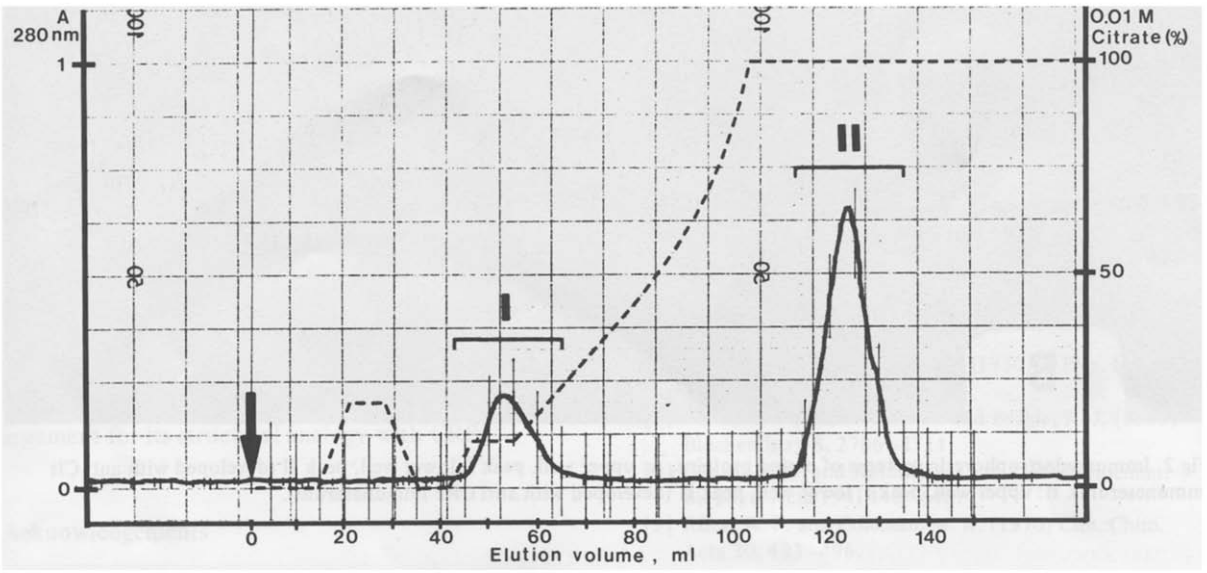
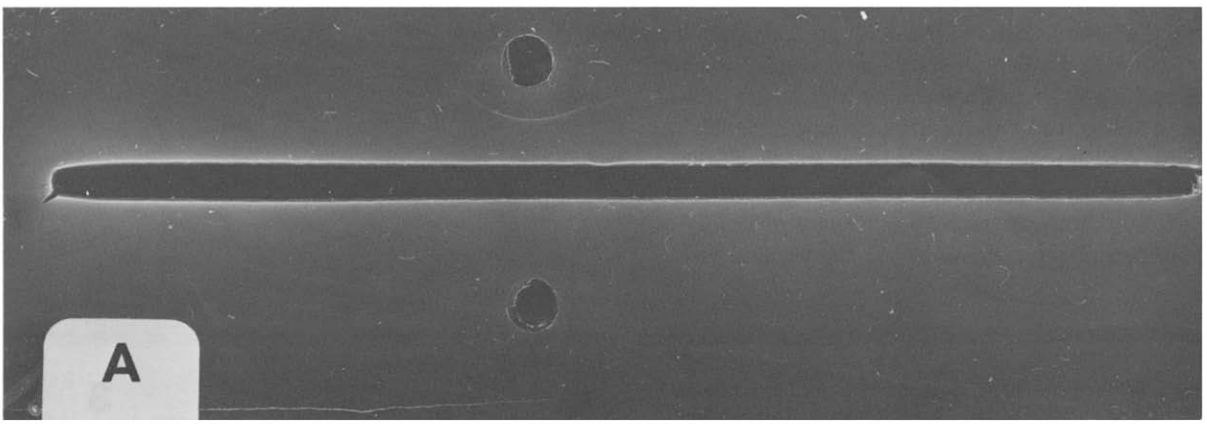


Fig.1. Affinity chromatography of inflammatory human serum. Conditions were: 30 ml gel bed, sample 20 ml. Elution system: 0.01 M citrate, gradient (– – – – – –) 0.02 Tris–HCl buffer, 0.1 M NaCl, pH 8. A_{280} (———).

tion operations were performed at 6°C, with a 10 ml/h flow rate. The eluate was monitored at 280 nm with a Gilson automatic ultraviolet scanner and collected by fractions of 5 ml.

3. Results and discussion

The affinity column was equilibrated with 0.02 M Tris–HCl buffer, 0.1 M NaCl, 0.01 $CaCl_2$, pH 8, for 24 h. Pooled inflammatory human serum (20 ml), containing more than 3 g/l haptoglobin, was filtered on 1.2 μm millipore filter, and applied on the column. Elution was started with the same buffer. When the A_{280} was less than 0.02, the column was washed with Ca^{2+}-free buffer, for 12 h, and absorbance increased weakly. Then a gradient (see fig.1) expressed as 0.01 M citrate percentage in the same buffer, eluted 2 well-separated peaks. Later on, a 0.02 M citrate washing (100 ml), did not bring about any increased absorbance. The column was then regenerated, as indicated. Material from peaks I and II was concentrated and analysed.



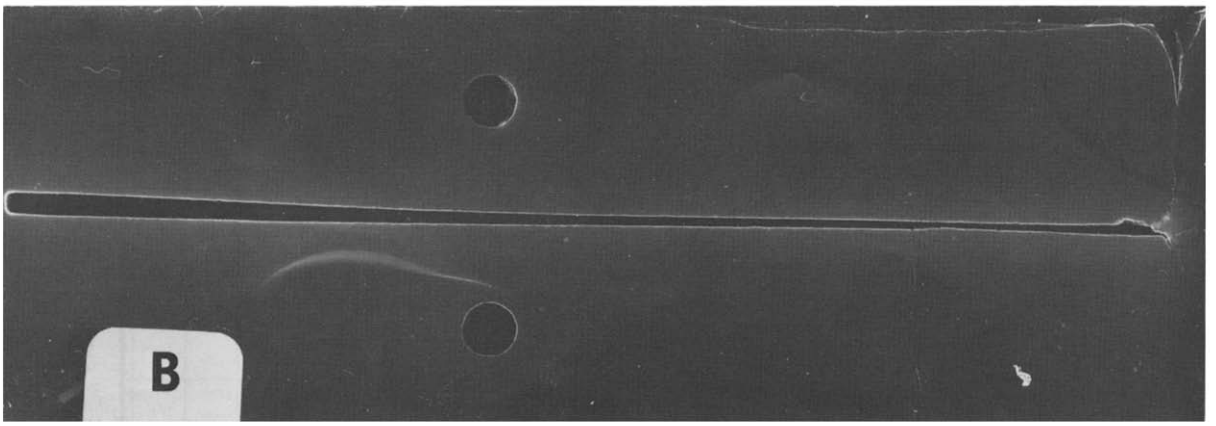


Fig.2. Immunoelectrophoretic patterns of eluted proteins. A: upper well, peak I; lower well, peak II (developed with anti CIt immuneserum). B: upper well, peak I; lower well, peak II (developed with anti CRP immuneserum).

The unique present protein revealed by immunoelectrophoresis in peak I was CIt (fig.2). No contaminant could be detected by using several monospecific antisera directed against human serum proteins.

Polyacrylamide gel electrophoresis revealed a band with a low mobility (fig.3). The peak II fraction, once concentrated, did not show any contaminant, when tested with an antiserum to whole normal human serum. Only a monospecific CRP antiserum revealed a γ mobility arc, identical to the CRP standard pattern (fig.2). In acrylamide gel, the same peak showed only 1 slow-moving band (fig.3). A pool of normal human serum treated alike did not present the second peak. Identical results have been repeatedly obtained at least 10 times with a single column. Calcium-free buffer was used for 2 major reasons. It washed off free calcium ions, and allowed the citrate gradient to chelate the bound calcium ions. Both proteins were then separated by gradient, that otherwise would have been mixed in a single peak. Secondly, this buffer proved to be efficient to remove weak-binding material. The yield of CRP preparation was never less than 70% and CRP was not detectable in the column effluent.

If this chromatography procedure is obviously calcium dependent, it does not point out which part of

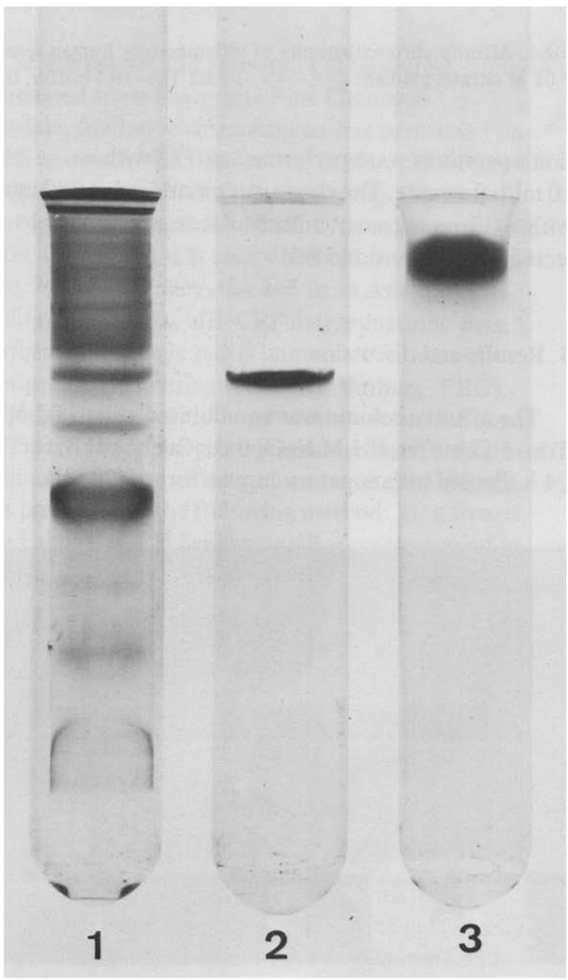


Fig.3. Polyacrylamide gel electrophoresis of human serum (1), CIt (2) and CRP (3) isolated by affinity chromatography.

the ligand is responsible for the binding. Activated CH Sepharose 4B is made of agarose, which is a complex substance, widely varying with different batches. Sepharose 4B treated in exactly the same conditions, showed a weak but significant CRP and CIt retention. Comparable fixation–elution procedures led to a less than 10% recovery. The ligand-coupled gel is therefore far more efficient than the bare Sepharose.

The proposed method to prepare CRP consists of a simple one-step procedure, that outdates the use of pneumococcal C-polysaccharide. Moreover, CIt, never prepared before with C-polysaccharide, is obtained in the course of the same process. This brings a new argument for its structural analogy with CRP.

Acknowledgements

We thank Professor M. G. Colomb for his generous gift of CIt and antisera and helpful discussion.

References

[1] Claus, D. R., Osmand, A. P. and Gewurz, H. (1976) J. Lab. Clin. Med. 87, 120–128.
[2] Osmand, A. P., Friedenson, B., Gewurz, H., Painter, R. H., Hofmann, T. and Shelton, E. (1977) Proc. Natl. Acad. Sci. USA 74, 739–743.
[3] Oliveira, E. B., Gotschlich, E. C. and Liu, T. H. (1977) Proc. Natl. Acad. Sci. USA 74, 3148–3151.
[4] Skinner, M., Benson, M. D. and Cohen, A. S. (1976) Arthritis Rheum. 19, 822 (Abstr.).
[5] Binette, P., Binette, M. and Calkins, E. (1974) Biochem. J. 143, 253–254.
[6] Tillet, W. S. and Francis, T., jr (1930) J. Exp. Med. 52, 561–571.
[7] Davis, R. V., Blanken, R. M. and Beagle, R. J. (1969) Biochemistry 8, 2706–2711.
[8] Ganrot, P. O. and Kindmark, C. O. (1969) Biochem. Biophys. Acta 194, 443–448.
[9] Riley, R. F. and Coleman, M. K. (1970) Clin. Chim. Acta 30, 483–496.
[10] Hokama, Y., Tam, R., Hirano, W. and Kimura, L. (1974) Clin. Chim. Acta 50, 53–62.