Journal of Chromatography, 87 (1973) 239–242

CHROM. 6955

Note

Modified agarose derivative for affinity chromatography

Application to purification of human α-fetoprotein

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(First received May 15th, 1973; revised manuscript received July 23rd, 1973)

There have been a number of published reports on the use of agarose derivatives. An elegant method has been described for coupling proteins to these derivatives by using cyanogen bromide[1–4] and the property of proteins to bind ligands specifically and reversibly is used in affinity chromatography for their selective isolation and purification.

This use may be limited by the low affinity or steric hindrance of high-molecular-weight proteins. Recently, another reported technique[5] described indirect fixation of proteins by cross-links between ligands and the agarose matrix; such a cross-link, a hydrocarbon chain of sufficient length, is shown to be of great importance for better coupling and for a more open structure of the immunoadsorbent, which reduces steric hindrance.

Here, phloroglucinol and epichlorhydrin are cross-linking agents.

This paper reports the preparation of this agarose derivative, and its application to human α-fetoprotein purification.

MATERIALS AND METHODS

Preparation of immunoadsorbents

Phloroglucinol–hydroxypropyl–agarose is prepared as follows. Sepharose 4B (100 ml; Pharmacia, Uppsala, Sweden) is mixed at room temperature with 15 g of phloroglucinol (E. Merck, Darmstadt, G.F.R.). The mixture is treated with 10 ml of epichlorhydrin (E. Merck) in 1 *M* NaOH (total volume equal to the volume of Sepharose) and heated to 50° with stirring for 2.5 h. The gel is transferred to a büchner funnel and washed under suction with distilled water and finally with a volume of 0.1 *M* $NaHCO_3$, this volume being 15 to 20 times that of the packed Sepharose.

A given ligand is coupled to the hydroxyl groups of the phloroglucinol–hydroxypropyl–agarose by activation with cyanogen bromide (Bromcyan, Schuchardt, München, G.F.R.) by the method of Porath *et al.*[3,4] as modified by Cuatrecasas *et al.*[6] The gel, suspended in an equal volume of distilled water, is added to finely divided cyanogen bromide (300 mg per ml of Sepharose) in a ventilated hood and at low temperature. The pH of the suspension is immediately raised to 11 and maintained at 11 by dropwise addition of 2 *M* NaoH to stabilization (about 8 min). The gel is quickly transferred to a büchner funnel containing crushed ice and washed with

15 vol. of cold 0.1 *M* $NaHCO_3$, then mixed in a beaker with the ligand dissolved in a volume of 0.1 *M* $NaHCO_3$ equal to the volume of packed Sepharose. The coupling is effected overnight with gentle stirring at 4°. The suspension is transferred again to a büchner funnel and washed sequentially with 15 vol. of 0.1 *M* $NaHCO_3$, 10 vol. of elution buffer (0.1 *M* glycine–HCl, pH 3) and 15 vol. of 0.08 *M* NaCl.

The amount of ligand bound to the adsorbent is calculated from the protein remaining in washings, or directly by determination of nitrogen per ml of gel.

Purification of human α-fetoprotein

For this application the ligand coupled to phloroglucinol–hydroxypropyl–agarose is the specific antibody to human α-fetoprotein obtained by fractionation from the antiserum (RA Hu/FP, Nordic Pharmaceuticals and Diagnostics, Tilburg, The Netherlands) with an equal volume of 50% saturated ammonium sulphate, equilibrated at 0°; the precipitate is dissolved in distilled water and dialyzed against distilled water. A 44-mg amount of antibody is coupled to 30 ml of adsorbent by the procedure described above. A good coupling is performed in 86% yield.

Antibody concentration is determined by radial immunodiffusion[7] using purified α-fetoprotein as a standard.

Then a batchwise adsorption on immunoadsorbent is carried out as follows. A 1200-ml amount of human ascitic fluid, containing 135 mg/l of α-fetoprotein, is added to the immunoadsorbent and stirred in a beaker for 16 h at 4°. The immunoadsorbent is decanted and washed with 0.08 *M* NaCl until the optical density at 280 nm of the eluate reaches a constant minimal value. The gel is then poured into a 1.6 × 30-cm column and the elution of the retained protein carried out by 0.1 *M* glycine–HCl, pH 3, with a flow of 1.2 ml/min; the fractions emerging from the column are immediately neutralized with 0.2 *M* tris–H_3BO_3 buffer, pH 8.6, and pooled in two parts concentrated by pressure dialysis in collodion bags.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram obtained. Two fractions are pooled separately according to analytical electrophoretograms of different tested portions of the peak eluted: I comprising the ascending and the middle parts, II the descending part of the peak.

The yield after adsorption is 70% for fraction I: 36 mg of α-fetoprotein is recovered, and the resulting preparation is pure when examined by the usual methods of electrophoresis and immunodiffusion. The purity of the preparation is further established by the monospecific antisera obtained in several rabbits upon immunization.

The calculated yield after adsorption is 15% for fraction II, which appears to consist of α-fetoprotein and small amounts of impurities: albumin and γ-globulins.

By using the column of immunoadsorbent repeatedly with the 1200 ml of ascitic fluid, 120 mg of α-fetoprotein are obtained.

In order to determine the advantage of interposition of phloroglucinol–hydroxypropyl between Sepharose and ligand, a comparative study was also made with CNBr-activated, but unsubstituted, Sepharose. The same batch of antibodies was used, and ascitic fluid was applied to the control column under identical conditions.

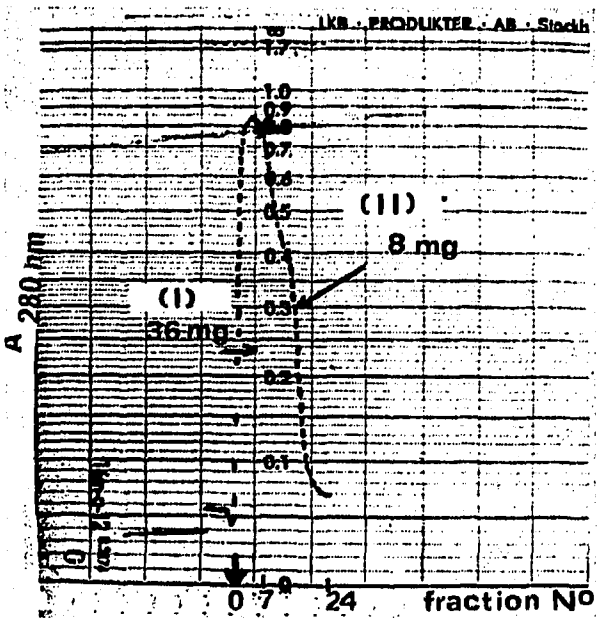

Fig. 1. Chromatogram obtained with human ascitic fluid after passage through a 1.6 × 30-cm column of phloroglucinol–hydroxypropyl–agarose coupled to human α-fetoprotein specific antibody. The experimental conditions are given in the text. Absorbance at 280 nm; chart speed, 10 mm/h; flow cell diameter, 3 mm; fraction volumes, 5 ml.

TABLE I

RECOVERIES OF α-FETOPROTEINS

Gel	*Total proteins (mg)* in ascitic fluid*	*α-Fetoprotein (mg)*			*Total proteins recovered (mg)*	*Purification as mg α-fetoprotein/g total protein*
		Applied	*Absorbed*	*Recovered*		
Sepharose 4B, unsubstituted	20,000	162	34	27	31	870
Phloroglucinol–hydroxypropyl–agarose	20,000	162	52	36 (I) 8 (II)	36 19	1000 421

* Protein determinations were made by the method of Lowry.

The recoveries of α-fetoprotein are shown in Table I. The present data suggest that yield and specificity are enhanced by cross-links of the type described. The advantages are (i) an increase, by the presence of a polyhydric phenol–phloroglucinol, in the total number of hydroxyl groups of higher reactivity available for ligand coupling (theoretically × 2), (a coupling yield of 86% suggests that it may be possible to prepare an immunoadsorbent containing a very high concentration of binding sites) and (ii) an increase in the distance between ligand and Sepharose matrix, which may make the binding sites after coupling more accessible to the protein.

A more open structure is also obtained, minimizing steric hindrance of large molecules. The stability of the immunoadsorbent is not changed by the cross-links and several experiments can be performed successfully.

In the particular example given, the preparation of this agarose derivative permits, compared with different published results[8,9], recovery of α-fetoprotein without impurities from reasonable amounts of human ascitic fluid.

REFERENCES

1 J. Porath, J. C. Janson and T. Laas, *J. Chromatogr.*, 60 (1971) 167.
2 P. Cuatrecasas, *J. Biol. Chem.*, 12 (1970) 3059.
3 J. Porath, R. Axen and S. Ernback, *Nature (London)*, 215 (1967) 1491.
4 R. Axen, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
5 J. Porath and L. Sundberg, *Nature (London)*, 238 (1972) 261.
6 P. Cuatrecasas, M. Wilchek and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.*, 61 (1968) 636.
7 G. Mancini and A. O. Carbonara, *Immunochemistry*, 2 (1965) 235.
8 S. Nishi and H. Hirai, *Biochim. Biophys. Acta*, 278 (1972) 293.
9 E. Alpert, J. Drysdale, K. Isselbacher and P. Schur, *J. Biol. Chem.*, 12 (1972) 3792.