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Note

Covalent coupling of cholic acid to aminohexylamino-Sepharose 4B and its use in affinity chromatography of serum albumin

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Conventional methods for removal or purification of albumin from serum have been generally disappointing. In more recent methods advantage has been taken of the ability of albumin to bind reversibly a variety of biological substances. The high affinity that albumin shows for bilirubin, fatty acids and certain organic dyes has led to the use of these compounds as ligands coupled to Sepharose for the separation of albumin from serum[1–3]. Bile acids are also bound by albumin[4] although use of this ligand has not yet been reported. This paper describes the synthesis of a cholic acid–aminohexylamino-Sepharose 4B gel, its serum albumin binding properties and its advantages over other affinity chromatography methods.

MATERIALS AND METHODS

Coupling of cholic acid to aminohexylamino-Sepharose 4B

^{14}C-Labelled cholic acid (40 mg; 1 μCi) was dissolved in 5 ml of absolute ethanol and added to 20 ml of 50% (v/v) aqueous ethanol containing 160 mg of 1 ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl. The pH of the solution was adjusted as required. The coupling solution was then slowly added to 2 ml of preswollen aminohexylamino(AH)-Sepharose 4B (Pharmacia, Uppsala, Sweden) and gently stirred at 24° for 16 h.

The substituted Sepharose was washed extensively on a Buchner funnel with 50% aqueous ethanol (pH 7.4) and 0.5 *M* NaCl (pH 7.4) until ^{14}C-labelled cholic acid was free from the effluent. Since the capacity of the column to bind albumin did not alter with the use of the column, it appears that washing was complete and that there was no leakage of coupled bile acid. The cholic acid coupled Sepharose was stored in buffer (20 m*M* phosphate; 0.5 *M* NaCl pH 7.4) at 4° in the presence of 0.1% sodium azide and was stable for periods greater than 3 months.

The bile acid content of the various gel preparations was estimated by counting an aliquot of gel slurry dispersed in 10 ml of Triton X-100–toluene scintillant in a Packard Tri-Carb scintillation counter (Model B2450).

Affinity chromatography

Bile acid–Sepharose preparations were packed in 5-ml polypropylene syringes,

plugged with glass wool (packed gel volume 2 ml). Solutions of bovine serum albumin (BSA) were passed through these columns at room temperature in buffer (20 mM phosphate, 0.14 M NaCl, pH 7.4) at a flow-rate of 30 ml/h, and further washed with 40 ml of the same buffer before elution of bound protein. Protein was measured by absorbance at 280 nm using BSA as standard. For re-use the columns were washed and equilibrated with phosphate–saline buffer.

The maximal capacity for uptake of BSA was measured by saturation of the column with a solution of the protein, washing with 40 ml of buffer and subtraction of the amounts of albumin in the effluent from the amount added.

Affinity chromatography of albumin from human serum was studied by passing 4 ml of 1:20 diluted serum sample through the column and washing as above.

Electrophoresis

Sodium dodecyl sulphate (SDS) gel electrophoresis was carried out in 7% polyacrylamide gels[5]. Quantitation of protein was done using a gel scanner fitted to a Beckman Spectrophotometer Model 25 at 280 nm and after staining with Coomassie Blue at 620 nm. Immunoelectrophoresis was carried out on 1% agarose gel plates before addition of whole human antisera (Dakopatts, Copenhagen, Denmark) to troughs running parallel to the samples. Precipitation lines were stained with Coomassie Blue.

RESULTS AND DISCUSSION

Coupling reaction

Cholic acid was coupled to AH-Sepharose 4B using ethyl(dimethylaminopropyl)carbodiimide, at various pH values (Table I). Maximal coupling of 3.3 μmol/ml swollen gel was obtained at pH 7.0.

TABLE I

EFFECT OF pH ON COUPLING OF CHOLIC ACID TO AH-SEPHAROSE 4B

pH	*Cholic acid coupled (μmol/ml packed gel)*
4	0.0
5	1.2
6	2.8
7	3.3
8	0.3

Binding and elution of albumin

Columns containing substituted or native AH-Sepharose 4B preparations were loaded with BSA and washed with buffer. Retention of albumin was specific to cholic acid coupled Sepharose. No BSA was retained by the native unsubstituted Sepharose. The maximal capacity of the gel for BSA was 0.044 μmol/ml of gel. Elution of BSA bound to Sepharose could be accomplished at pH 7.4 by 8 M urea, or under non-denaturing conditions by competitively eluting with 5 mM cholic acid or 5 mM taurocholic acid.

Purification of albumin from human serum

When diluted human serum was added to the affinity column, immunoelectrophoretic studies of the eluent showed that nearly all of the albumin had been bound on the gel. There was some minor binding of other serum proteins but these also bound to the unsubstituted control Sepharose. These non-specifically bound proteins could be removed almost entirely by elution with 0.5 *M* NaCl, but this also resulted in a greater than 50% loss of bound albumin. The remaining bound albumin could be eluted by 8 *M* urea or 5 m*M* bile acid (Fig. 1B).

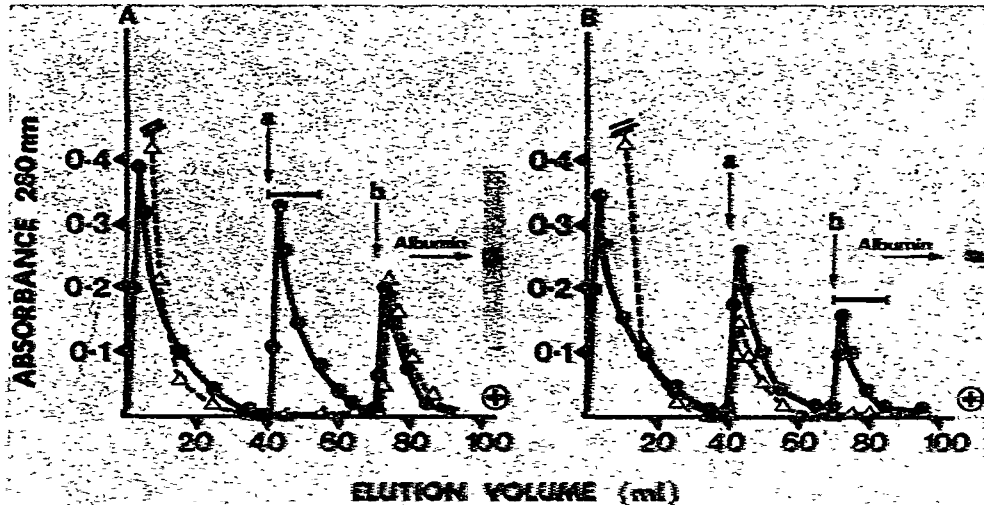

Fig. 1. Affinity chromatography of albumin from human serum. 4 ml of dilute (1:20) serum was applied to both substituted (●—●) and native (△—△) columns (packed gel volume 2 ml) and washed immediately with phosphate–saline buffer (pH 7.4). (A) Bound albumin was specifically eluted with (a) 5.5 m*M* cholic acid (pH 7.4). Non-specifically bound serum protein which remained on the column was removed by (b) either 0.5 *M* NaCl (pH 7.4) or 8 *M* urea (pH 7.4). (B) Non-specifically bound protein together with 40–60% albumin was eluted with (a) 0.5 *M* NaCl (pH 7.4). The remaining albumin bound with higher affinity was subsequently removed by (b) 8 *M* urea or 5 m*M* cholic acid (pH 7.4). The albumin peak indicated by the horizontal bar was pooled, concentrated and analysed for purity by SDS–polyacrylamide electrophoresis, run in the presence of mercaptoethanol.

In contrast, specific elution with 5 m*M* cholic acid gave complete recovery of albumin uncontaminated with other serum proteins (Fig. 1A). Non-specifically bound protein remained bound to the gel. Purity of the eluted albumin was assessed by SDS–polyacrylamide electrophoresis. Scanning of the SDS gels at 280 nm and at 620 nm after staining with Coomassie Blue showed the purity of the eluted albumin to be greater than 99%. The ability of the bile acids (cholic acid and taurocholic acid) to elute albumin indicates that the binding of albumin to the gel is through a specific bile acid binding site. Elution of albumin by bile acids is not due to their general detergent properties since non-specifically bound serum protein on both substituted and native Sepharose remained bound to the gel in the presence of 5 m*M* cholic acid.

The cholic acid–Sepharose gel has proved useful for the purification of albumin from serum. Although the capacity of the gel for albumin (3 mg/ml swollen gel) is not

as high as that reported for other affinity methods[1–3], it does have a number of advantages. The technique is simple and allows elution of albumin (>99% pure) in one step, in a non-denatured form. The matrix is very stable and the ligand readily coupled. None of the other methods reported has all of these advantages. Since the various ligands appear to take advantage of different binding sites[6,7], cholic acid coupled Sepharose may prove a useful tool in studying binding sites on albumin. The gel has already proved useful in the isolation of receptor/carrier proteins involved in the enterohepatic transport of bile acids[8].

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