PROCION RED HE-3B EXTRACTS PLASMINOGEN FROM HUMAN SERUM

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Received 2 May 1979

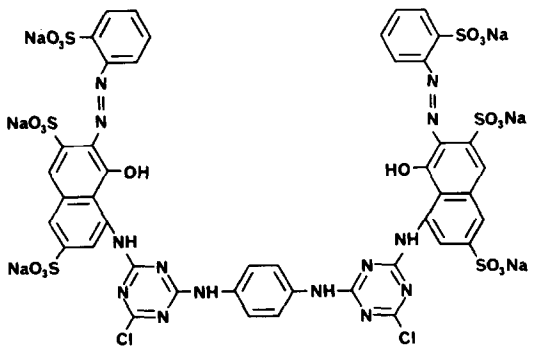
1. Introduction

Several synthetic dyes immobilised on solid supports have been examined for the affinity chromatography of proteins [1,2]. In particular, extensive use has been made of Cibacron blue F3GA as a ligand with some specificity for NADH-dependent enzymes [1]. Recently, another dye, Procion red HE-3B (fig.1), has been recommended for chromatographing NADPH-dependent enzymes [3]. As Cibacron blue affinity columns have been found to specifically extract albumin from human serum [4] this prompted us to investigate the use of Procion red HE-3B as an affinity ligand for serum proteins.

2. Methods

2.1. *Synthesis of Procion Red HE-3B–Sepharose-4B*

Sepharose-4B (Pharmacia) was washed several times with water on a sintered glass funnel and dried to a moist cake, 25 g gel were then suspended in water containing 2.5 g NaCl and 250 mg Procion red HE-3B (ICI Ltd, Manchester) and made up to 45 ml. After 30 min of end-over-end mixing, 5 ml of a 1% (w/v) solution of NaOH was added to the mixture and mixing continued overnight at room temperature. The now red-dyed Sepharose-4B (red gel) was recovered by filtration and washed copiously, first with water and then with phosphate buffer (0.05 M (pH 7.4) containing 1 mg/l sodium azide). The red gel was stored in the buffer at 4°C where no leaching of dye was observed over 6 months.



Procion red HE3B

Fig.1. The structure of Procion red HE-3B. One or both of the chlorotriazinyl groups react with hydroxyl on Sepharose to form an ether linkage. An unreacted chlorotriazinyl group hydrolyses to hydroxytriazinyl.

2.2. *Synthesis of lysine–Sepharose-4B*

Lysine was coupled through its α-amino group to Sepharose-4B by reacting N-*tert*-butoxycarbonyl lysine (8 mg) with 1 g CNBr-activated Sepharose (Pharmacia) in 5 ml dimethyl formamide – 0.1 M $NaHCO_3$/1 M NaCl, 1:1 (v/v) overnight at room temperature. After washing as recommended by the manufacturer, the gel was suspended in water to give 3 ml a total vol., 7 ml trifluoroacetic acid were added and the mixture agitated for 2 h at room temperature to remove the *tert*-butoxycarbonyl group. The gel was then washed copiously with water. The presence of free amino groups was established using ninhydrin.

3. Results

Pooled human serum (25 ml) was applied to a 1.5 × 30 cm column of red gel pre-equilibrated with 0.05 M phosphate buffer (pH 7.4) and washed through with this buffer until the eluate A_{280} had fallen to a constant value (typically 0.05 A_{280}).

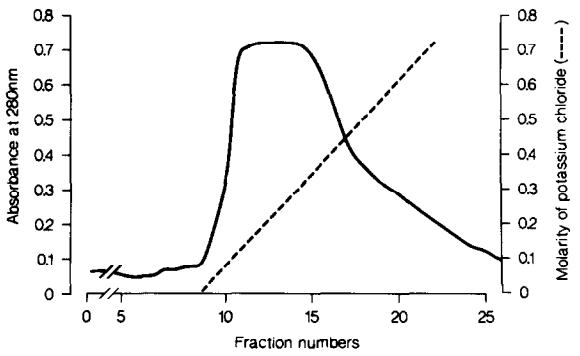


Fig.2. Gradient elution of protein adsorbed from pooled serum (25 ml) onto a column of Procion red HE-3B–Sepharose-4B (1.5 × 30 cm). Fractions (4.7 ml) were collected; A_{280} was continuously recorded (——) and the concentration of KCl is shown (– – – – – –).

Adsorbed protein was eluted with 200 ml of a 0–2 M KCl gradient made in the phosphate buffer. Eluted material was monitored continuously at 280 nm. Fractions (4.7 ml) were collected and the potassium concentration was determined by flame photometry. The single peak of ultraviolet absorbent material was eluted at 0.15 M on the KCl gradient (fig.2). The fractions below the central area of the eluted peak were pooled (typically 30 ml) and dialysed against 0.01 M ammonium acetate at 4°C for 24 h. The protein was recovered by freeze drying, reconstituted in buffer and stored frozen.

Two-dimensional immunoelectrophoresis [5] using anti-whole human serum (Wellcome) showed one large peak in the region of β serum proteins.

Analysis of the protein by 'rocket' immunoelectrophoresis [6] using antisera (Behringwerke) specific for caeruloplasmin, fibrinogen, haemopexin, transferrin and IgG gave negative results but a positive result was obtained on a plate specific for plasminogen.

Gel filtration studies of the protein using a calibrated column of Sepharose-6B (1.6 × 80 cm) eluted with 0.05 M phosphate buffer (pH 7.4) revealed one protein peak only with mol. wt ~85 000 and some high molecular weight material constituting < 0.2% of the total protein.

Dansylation of the protein according to [7] gave glutamic acid as the only end group when analysed on polyamide sheets as described [8].

These results are consistent with the protein being plasminogen which is a β-glycoprotein with mol. wt 81 000–92 000 [9] and with a single amino terminal residue of glutamic acid [9]. In some reports lysine has been found to be the N-terminal residue but it has been suggested that this is a partially degraded form [9].

As additional confirmation, the protein's enzymic properties were examined. Plasminogen is not active until it has been converted by a kinase to plasmin which is a protease with specificity for bonds with the carboxyl group of lysine or arginine. In agreement with this, the red gel protein hydrolysed α-casein [10] only when streptokinase was included in the incubation mixture.

Using rocket immunoelectrophoretic analysis of eluted fractions a 5 ml column of red gel was shown to absorb all the plasminogen from a maximum of 7 ml serum. When this column was eluted with 0.2 M KCl in 0.05 M phosphate buffer (pH 7.4), 1.6 mg plasminogen was recovered (Folin-Lowry protein analysis [11]); equivalent to 23 mg/100 ml original serum.

The affinity gel lysine–Sepharose has been shown to extract plasminogen from serum [12]; the zymogen can then be eluted with 6-aminohexanoic acid. Plasminogen prepared in this fashion was subsequently absorbed by a column of red gel and could be eluted with KCl as above. Plasminogen could not be eluted by 6-aminohexanoic acid from the red gel thus indicating that different sites on the protein are employed in binding to each affinity gel.

4. Discussion

The use of affinity chromatography in the purification of proteins often obviates the need for the multi-step classical approach and is an obvious choice in a protein purification scheme. The synthetic dye, Procion red HE-3B, when immobilised on Sepharose has been shown to exert a class-specific adsorption for NADPH-dependent enzymes [3]. We have shown that, under the conditions used, Procion red HE-3B also exhibits a high specificity for human plasminogen.

Other affinity gels using lysine [12] or butyl *p*-amino benzoate [13] as immobilised ligands have been employed for the isolation of plasminogen. Synthesis of these absorbants is, however, complicated

and involves the hazardous use of cyanogen bromide; alternatively they are expensive to buy. Procion red HE-3B–Sepharose-4B is by contrast, cheap and simple to prepare and its use gives a one step procedure for the isolation of plasminogen.

Acknowledgement

We thank Imperial Chemical Industries Ltd for the generous gift of Procion red HE-3B.

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