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Note

Haeme–Sepharose 4B as a chromatographic matrix for the isolation of haemopexin from human serum

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We have previously described[1] the preparation of an affinity column with haeme attached to Bio-Gel P-200 and its application to the isolation of haemopexin from human serum. The procedure was considerably simpler than earlier methods based on precipitation and chromatographic techniques[2–7] and yielded haemopexin with a recovery of 20%. Recently an elegant procedure was reported[8] in which wheat germ lectin–Sepharose was used for the separation of haemopexin from transferrin. The method gives a yield of haemopexin of 37%, but the chromatographic matrix is very expensive. In this paper, a method for the attachment of haeme to Sepharose 4B is described and the application of a haeme–Sepharose 4B column to the isolation of haemopexin from human serum with a recovery of 80% is presented.

MATERIALS AND METHODS

Cyanogen bromide-Sepharose 4B and DEAE-Sepharose CL-6B were products of Pharmacia (Uppsala, Sweden) and 3,3′-diaminodipropylamine was obtained from Fluka (Buchs, Switzerland). The 3-N-*m*-nitrobenzoyl-3,3′-diaminodipropylamine (NBDD) was prepared as follows. Into a three-necked 250-ml flask equipped with stirrer, reflux condenser and funnel, cooled with water, were placed 13.1 g (0.1 mole) of 3,3′-diaminodipropylamine dissolved in 80 ml of benzene. Over a period of 1 h 18.6 g (0.1 mole) of *m*-nitrobenzoyl chloride dissolved in 80 ml benzene were added from the funnel with continuous stirring. A precipitate of 3-N-*m*-nitrobenzoyl-3,3′-diaminodipropylamine hydrochloride was formed, which was recrystallized from ethanol–water–triethylamine (3:1:1) to yield the free base.

Preparation of Sepharose 4B with covalently bound haeme

A 15-g amount of cyanogen bromide-Sepharose 4B was activated by washing with 2 l of 0.001 *N* hydrochloric acid and suspended in dimethylformamide (DMF)–water (1:1) containing 0.1 *M* sodium hydrogen carbonate. Then 25 mg of NBDD in 20 ml of DMF–water (1:1) were added and the mixture was shaken gently at room temperature for 2 h. The nitro derivative was reduced for 40 min at 40° with 0.1 *M* sodium dithionite in 0.5 *M* sodium hydrogen carbonate solution at pH 8.5. The amino derivative was diazotized for 7 min at 0–4° with a 0.1 *M* solution of sodium nitrite in 0.5 *M* hydrochloric acid. To the diazo derivative 50 mg of haemin in saturated borax solution were added and the reaction was allowed to proceed overnight with

gentle shaking. After each step the gel was washed thoroughly with water. The mode of attachment of haeme by a propionyl group to the gel matrix is shown in Fig. 1.

A gel column of dimensions 26 × 55 mm was prepared and haeme that remained non-covalently attached to the gel matrix was released by elution with 1% albumin in 0.1 *M* sodium phosphate solution (pH 7.0). As a proportion of the albumin molecules interacted with covalently bound haeme during this procedure, they were released from binding by elution with 0.35 *M* glycine–hydrochloric acid buffer (pH 2.4). Subsequently, the gel was re-equilibrated with 0.1 *M* sodium phosphate solution (pH 7.0).

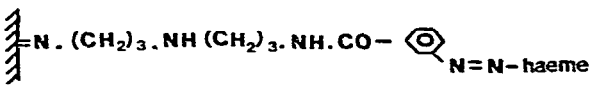

Fig. 1. Attachment of haeme to Sepharose gel matrix.

Preparation of crude glycoprotein fraction

Human serum contains two proteins with a high affinity for haeme, namely haemopexin and albumin[9,10]. Of these, it is of interest to isolate haemopexin by affinity chromatography as no simple conventional procedure has been described for its isolation in high yield. To remove albumin, which would otherwise compete for reactive centres on an affinity matrix, precipitation of human serum was first performed with rivanol (6,9-diamino-2-ethoxyacridine-lactate). To one volume of human serum at pH 8.0 an equal volume of 1.68% rivanol solution of pH 8.0 was added dropwise at 5°. Precipitated albumin was separated by centrifugation and from the supernatant containing haemopexin, transferrin, other serum glycoproteins and immunoglobulins dissolved rivanol was removed by precipitation with 5% sodium chloride followed by filtration through Schleicher & Schüll No. 597 1/2 filter-paper and passage through a Sephadex G-25 column equilibrated with 5% sodium chloride. The protein fraction that was eluted in approximately double the volume of serum originally taken and that contained haemopexin at a concentration of 0.3–0.4 g/l was dialysed against 0.1 *M* sodium phosphate solution (pH 7.0).

Analytical procedures

The purity of the protein fractions was checked by disc electrophoresis in Tris–glycine buffer (pH 8.4) and by immunoelectrophoresis against horse anti-human serum proteins, anti-haemopexin, anti-IgC, anti-albumin and anti-transferrin sera produced by Sevac (Prague, Czechoslovakia). The concentration of haemopexin was determined by radial immunodiffusion employing M-Partigen anti-haemopexin immunodiffusion plates and standard human serum, both products of Behringwerke (Frankfurt/Main, G.F.R.).

RESULTS AND DISCUSSION

Affinity chromatography of haemopexin

A 25-ml volume of the glycoprotein fraction was applied on the haeme–Sepharose 4B column (26 × 55 mm) at a flow-rate of 60 ml/h and the unbound

proteins were washed out with 0.1 *M* sodium phosphate solution (pH 7.0) (Fig. 2, peak 1) with monitoring on a Uvicord II instrument (LKB, Stockholm, Sweden) at 280 nm at a flow-rate of 150 ml/h (if monitoring is performed at 254 nm, a baseline shift occurs on switching to glycine–hydrochloric acid buffer). When the absorbance had decreased to zero the proteins bound to the haeme–gel matrix were eluted with 0.35 *M* glycine–hydrochloric acid buffer (pH 2.4) (Fig. 2, peak 2). The protein material that was eluted with the acid buffer was immediately neutralized with 1 *N* sodium hydroxide solution and the column was re-equilibrated with sodium phosphate buffer (pH 7.0). In this manner four chromatographic cycles could be performed in a working day.

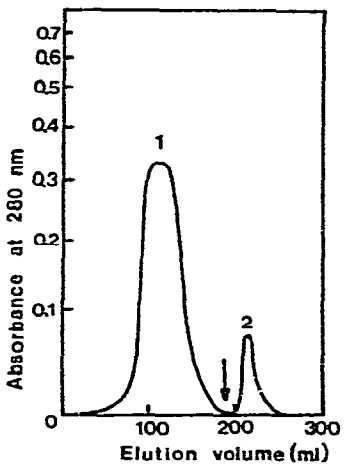

Fig. 2. Isolation of haemopexin from the rivanol supernatant of human serum on a haeme–Sepharose 4B column. Bed volume, 30 ml; 0.1 *M* phosphate buffer (pH 7.0). Glycine–hydrochloric acid buffer (pH 2.4) was applied at the point indicated by the arrow. The absorbance was monitored on a Uvicord instrument at 280 nm. Peaks: 1, unbound proteins; 2, affinity bound proteins.

In addition to haemopexin, this fraction also contained substantial amounts of immunoglobulins (*ca.* 30%), probably resulting from their interaction with the spacer groups. For their removal a further chromatographic step was necessary.

Purification of haemopexin fraction on DEAE-Sepharose CL-6B

The product of 1 week's affinity chromatographic experiments containing 150 mg of haemopexin contaminated with immunoglobulins was dialysed against 0.01 *M* sodium phosphate solution (pH 7.0). All of the material was applied on a column of DEAE-Sepharose CL-6B (16 × 100 mm) equilibrated with 0.01 *M* sodium phosphate solution (pH 7.0) and eluted first with the same buffer at a flow-rate of 40 ml/h with monitoring at 280 nm. After all the immunoglobulins had been eluted in a volume of *ca.* 80 ml (Fig. 3, peak 1), the haemopexin that remained on top of the DEAE-Sepharose column at this ionic strength was eluted with 0.1 *M* sodium phosphate solution (pH 7.0) in a volume of 30 ml (Fig. 3, peak 2). Haemopexin was then concentrated to a concentration of 2 mg/ml by ultrafiltration on an Amicon cell employing a Diaflo UM 10 membrane and 2-ml aliquots were stored deep-frozen.

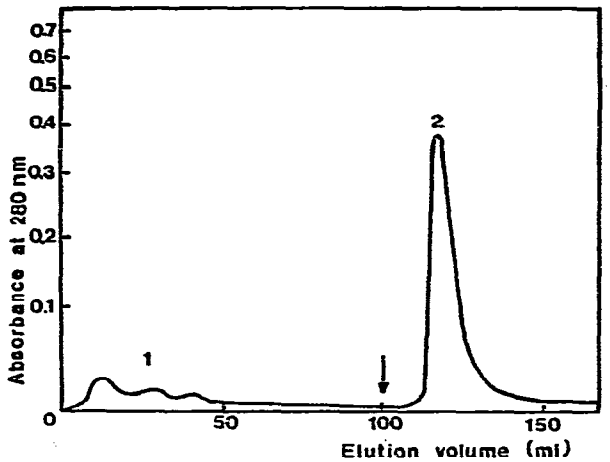

Fig. 3. Purification of haemopexin fraction on DEAE-Sepharose CL-6B. Bed volume, 20 ml; 0.01 *M* phosphate buffer (pH 7.0). The 0.1 *M* phosphate buffer (pH 7.0) was applied at the point indicated by the arrow. The absorbance was monitored on a Uvicord instrument at 280 nm. Fraction 1, immunoglobulins; peak 2, haemopexin.

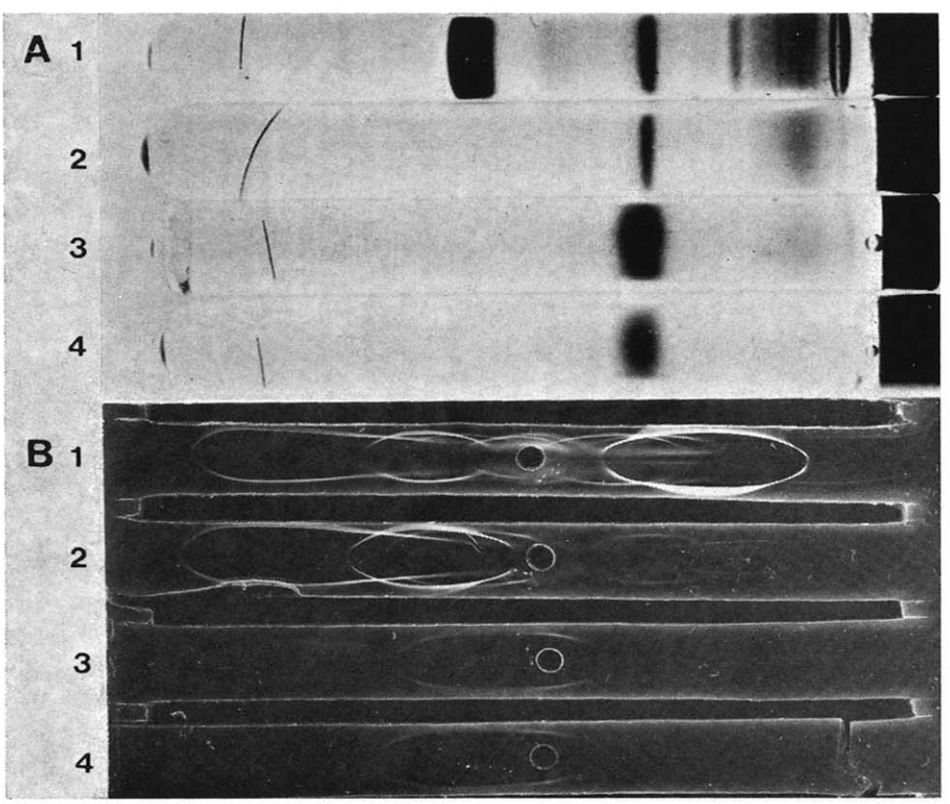

Fig. 4. (A) Acrylamide gel electrophoresis at pH 8.3 and (B) immunoelectrophoresis at pH 8.6 of (1) human serum, (2) rivanol supernatant of human serum, (3) haemopexin fraction isolated by affinity chromatography and (4) haemopexin after purification on DEAE-Sepharose CL-6B.

The amount of haemopexin obtained with the above column was 138 mg from 250 ml of serum, which corresponded to a recovery of 80%.

The haemopexin was pure on acrylamide gel electrophoresis and immunoelectrophoresis, as shown in Fig. 4, which compares the electrophoretic patterns of the rivanol supernatant of human serum, a haemopexin fraction obtained by affinity chromatography and haemopexin after final purification on DEAE-Sepharose CL-6B.

It is concluded that the haeme–Sepharose 4B gel matrix described here is a convenient medium for the isolation of haemopexin from human serum in high yield and purity. It can probably also be applied to the isolation of other proteins involved in haeme metabolism.

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