CHROM. 6165

Purification of human serum hemopexin by chromatography on DEAE-cellulose

Hemopexin is the serum β_1-glycoprotein with high affinity for heme, and its biological and physicochemical properties have recently been reviewed by MULLER-EBERHARD[1]. The detailed characterisation of the protein is hampered by the complexity and low yields of the isolation procedures[2-6]. The isolation method reported by HEIDE *et al.*[4] involves the use of precipitation of human serum with rivanol, ammonium sulphate and ethanol (or perchloric acid) followed by zone electrophoresis on a Pevicon block. As zone electrophoresis did not give reproducible results and no separation of hemopexin from transferin has been achieved, we replaced the final purification step by chromatography on DEAE-cellulose.

Materials and methods

Isolation of crude hemopexin fraction. Semipurified hemopexin was isolated by the method of HEIDE *et al.*[4] with modifications aimed to speed up the procedure. To 200 ml of serum (pH 9.4), 200 ml of 1.68 % rivanol solution (pH 6.5) were added dropwise at 5°. The precipitate was removed by centrifugation for 30 min at 30000 *g* at 5°. Rivanol was precipitated by the addition of solid NaCl to make a 5 % concentration and removed by filtration. To remove remaining traces of rivanol, the supernatant was passed through a Sephadex G-25 column (bed size 4 × 12 cm) equilibrated with 5 % NaCl. The eluate was dialysed overnight against 2 *M* $(NH_4)_2SO_4$ of pH 7.0. The precipitate formed on dialysis was removed by centrifugation at 30000 *g* and ammonium sulphate was removed from the supernatant by passage through a Sephadex G-25 column (bed size 8 × 80 cm) equilibrated with 0.15 *M* NaCl. The eluate was concentrated on an Amicon ultrafiltration cell to a volume of 50 ml and 0.5 ml of heme solution (1 mg/ml) was added. The solution was adjusted to pH 5.2 with 1 *M* acetic acid and cooled to 2°. Then 25 ml of ethanol cooled to −20° were added dropwise and the mixture was maintained at −13° for 10 min. The precipitate was removed by centrifugation at 30000 *g* for 30 min at −13°. The supernatant was dialysed overnight against 0.01 *M* sodium phosphate of pH 7.0 and concentrated to a volume of 10 ml on an Amicon ultrafiltration cell.

Chromatography on DEAE-cellulose. A 10-ml volume of the semipurified hemopexin fraction (protein concentration *ca.* 5 mg/ml) in 0.01 *M* sodium phosphate of pH 7.0 was applied to a 1.6 × 30 cm column packed with DEAE-cellulose (Whatman DE-11, capacity 1.0 mequiv./g). The sample was followed by 30 ml of the starting buffer, then by a linear gradient from 0–0.2 *M* NaCl in 0.01 *M* sodium phosphate of pH 7.0. The volume of the gradient was 700 ml. The flow-rate of 60 ml/h was maintained with the aid of a pump and 10-ml fractions were collected. The absorbance of the fractions was measured at 280 and 414 nm and the gradient was checked by conductivity measurements. The chromatography was carried out at 4°.

Fractions belonging to the individual chromatographic peaks were pooled, dialysed against 0.05 *M* sodium phosphate of pH 7.0 and concentrated on an Amicon ultrafiltration cell and Sartorius collodion membranes to a volume of 5 ml; the final protein concentration was 1–3 mg/ml.

Quantitation and examination of purity. The total protein concentration of the pooled fractions was determined by the method of Lowry. The concentration of hemopexin was ascertained by spectral measurement at 280 and 414 nm with $E_{1\,cm}^{1\,\%}$ values of 26.4 and 23, respectively[7], and by radial immunodiffusion[8] on Partigen®–Hemopexin immunodiffusion plates produced by Behringwerke AG, Marburg-Lahn, G.F.R. The concentrations of transferin and albumin were determined by radial immunodiffusion on Partigen–Transferin and Partigen–Albumin immunodiffusion plates.

Disc electrophoresis was performed by the method of DAVIES[9] in Tris–glycine buffer of pH 8.4.

Immunoelectrophoresis was carried out according to the method of SCHEIDEGGER[10] in barbital buffer of pH 8.4. Horse anti-human serum, rabbit anti-human transferin, anti-human albumin and anti-human haptoglobin sera were purchased from Sevac, Prague, Czechoslovakia, goat anti-human orosomucoid serum from Immunology Inc., Glen Ellyn, Ill., U.S.A., and rabbit anti-human hemopexin serum was kindly provided by Mr. KOŘÍNEK of this institute.

Results and discussion

The preparation of semipurified hemopexin resolves on DEAE-cellulose into four peaks, denoted I to IV (Fig. 1). Of these fractions, only fraction II absorbs significantly at 414 nm, indicating that hemopexin is present mainly in this eluate. Fig. 2 shows disc electrophoresis (A) and immunoelectrophoresis with horse anti-human serum in the troughs (B), of fractions I–IV. In disc electrophoresis, fractions I and II show the main band in equal positions corresponding to transferin and hemopexin, respectively (these two proteins have equal mobilities in acrylamide gel electrophoresis at pH 8.4). Only fraction II, however, gives an intensely coloured reaction with benzidine, which is a characteristic reaction of hemoproteins. Immunoelectrophoresis and radial immunodiffusion proved that fraction I consists mainly of

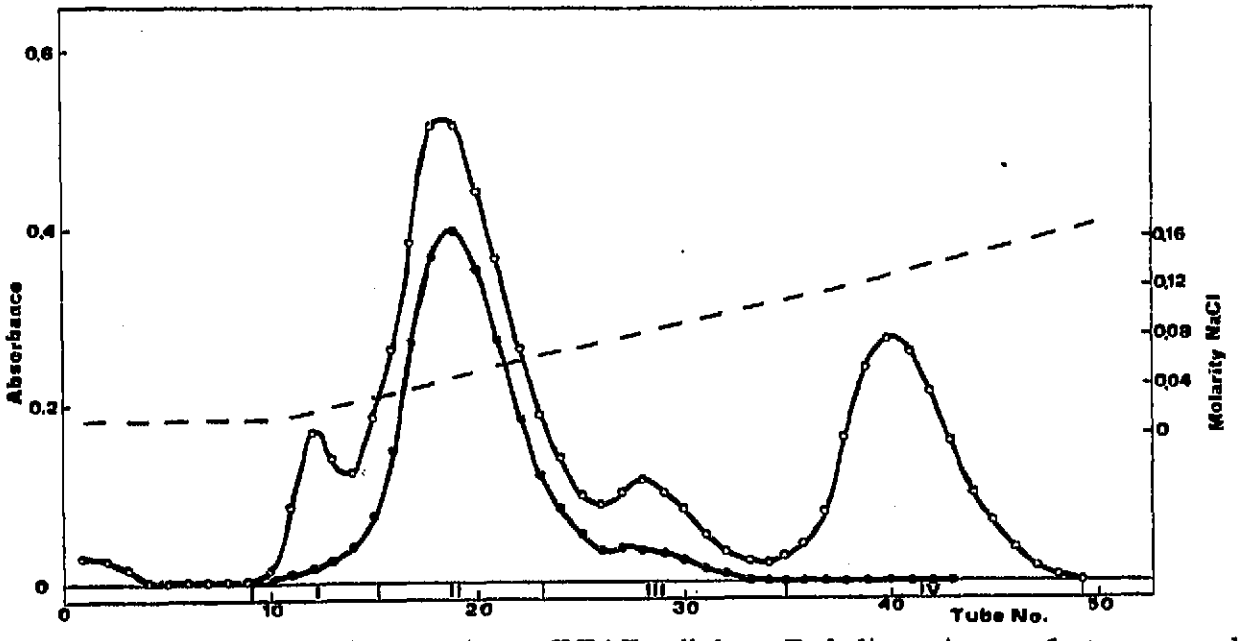

Fig. 1. Elution of crude hemopexin on DEAE-cellulose. Bed dimensions, 1.6 × 30 cm; eluant, 0.01 *M* sodium phosphate of pH 7.0 with a linear gradient to 0.2 *M* NaCl. ○, Absorbance at 280 nm; ●, absorbance at 414 nm.

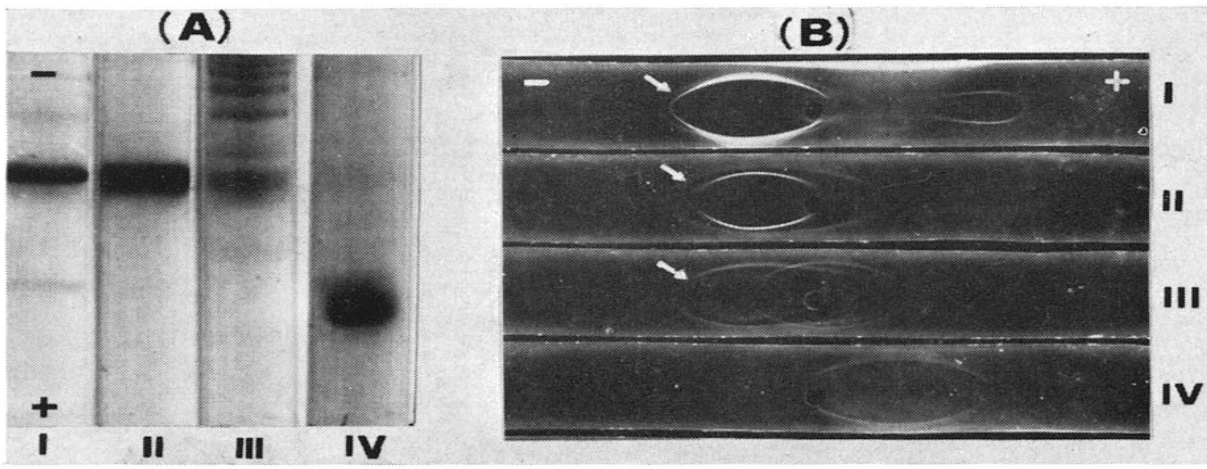

Fig. 2. Disc electrophoresis at pH 8.4 (A) and immunoelectrophoresis (B) of the DEAE-cellulose column fractions I–IV. Horse anti-human serum was used for the immunoprecipitation reaction. The precipitation line of hemopexin is indicated by arrows.

transferin contaminated with hemopexin while fraction II consists of hemopexin contaminated with transferin. On acrylamide gel fraction III gives one benzidine-positive band of hemopexin and a series of bands corresponding to a single protein and to its aggregated forms. Immunoelectrophoresis reveals, apart from hemopexin, a precipitation line in the region of α_2-globulins yielding a coloured reaction on carbohydrate staining. The assumption that this protein is haptoglobin was disproved by its negative reaction with monospecific antiserum as well as by its inability to bind hemoglobin. The protein of fraction IV belongs, judging from the position of the immunoprecipitation line, to the α_1-globulins. It gives a positive reaction on carbohydrate staining, but does not react with specific anti-orosomucoid serum. Apart from this component, traces of albumin in fraction IV have been proved with specific anti-albumin serum. The composition of the individual pooled fractions as well as quantitation of their compounds are given in Table I.

TABLE I

COMPOSITION OF THE DEAE-CELLULOSE COLUMN FRACTIONS

A 50-mg amount of crude hemopexin preparation was applied.

Fraction	*Molarity of NaCl*	*Components*	*Content (mg)*	*Relative content in the fraction (%)*
I	0.02	Hemopexin	0.3	5
		Transferin	6	95
II	0.04	Hemopexin	12	90
		Transferin	1.3	10
III	0.08	Hemopexin	0.8	10
		α_2-Globulin (unidentified)	7	90
IV	0.125	α_1-Globulin (unidentified)	22.6	100
		Albumin	(traces)	

Chromatography on DEAE-cellulose is a suitable method for the purification of crude hemopexin preparations. A significant advantage is the ability to remove most of the contaminating transferin, which is not readily attainable by zone electrophoresis or gel chromatography methods. The 90% purity of the resulting hemopexin preparation is sufficient for many physicochemical measurements. For purposes requiring higher purity, the middle portion of the chromatographic fraction II may be used.

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