The Use of Wheat-Germ Lectin–Sephacrose for the Purification of Human Haemopexin

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Haemopexin was prepared in 37% yield from normal human serum by a simple procedure involving fractional poly(ethylene glycol) precipitation and subsequent chromatography on DEAE-Sephacrose CL-6B. One peak from the ion exchanger consisted of only haemopexin and transferrin. These proteins were separated by chromatography on wheat-germ lectin–Sephacrose 6MB. Haemopexin was selectively bound and was subsequently desorbed by *N*-acetyl-D-glucosamine. No impurities could be detected in the final preparation by immunoelectrophoresis or by immunodiffusion against a range of antisera. The protein gave two partially separated bands in polyacrylamide-gradient-gel electrophoresis, corresponding to apohaemopexin and haem–haemopexin complex.

Haemopexin is a glycoprotein that is synthesized in the liver and is present in human serum in a concentration of 0.5–1.0 mg/ml (Muller-Eberhard, 1970). It has a mol.wt. of 57000 (Seery *et al.*, 1972) and consists of a single polypeptide chain, to which two carbohydrate chains, each of mol.wt. 5500, are attached (Muller-Eberhard & Morgan, 1975). The protein binds to haem and other porphyrins in an equimolar ratio and is responsible for the transport of haem to the liver. The haemopexin concentration in the blood depends on the concentration of free haem, and monitoring of serum haemopexin thus provides a valuable screening test for assessment of the severity of cardiac haemolysis (Eyster *et al.*, 1972).

The purification of haemopexin has always been difficult, which is probably the reason why the protein has not been studied very much (Muller-Eberhard & Morgan, 1975). Purification methods based on preparative electrophoresis (Muller-Eberhard & English, 1967) and ion-exchange chromatography (Hrkal *et al.*, 1972) gave low yields. Hayem-Lévy & Havez (1973) used a procedure involving rivanol and $(\text{NH}_4)_2\text{SO}_4$ precipitation and recycling chromatography on Sephadex G-100 to obtain a yield of 150–200 mg of haemopexin/litre of serum. Aisen *et al.* (1974) obtained almost pure haemopexin, mostly in dimeric form, in 30% yield from Cohn fraction IV-7 by ion-exchange and gel-filtration chromatography. Suttner *et al.* (1977) reported the isolation of human haemopexin in 20% yield by affinity chromatography on a haem-polyacrylamide conjugate, prepared by a seven-step synthesis.

In the present paper we describe a simple alternative method for the purification of haemopexin, based on the use of poly(ethylene glycol) precipitation,

ion-exchange chromatography on DEAE-Sephacrose CL-6B and affinity chromatography on wheat-germ lectin–Sephacrose 6MB. The haemopexin was analysed by polyacrylamide-gradient-gel electrophoresis, immunoelectrophoresis and double immunodiffusion. The amino acid content of the protein was determined and the results were compared with those of other investigators.

Experimental

Materials

Fresh serum from normal human individuals was obtained from the Academic Hospital, Uppsala, Sweden. DEAE-Sephacrose CL-6B, wheat-germ lectin–Sephacrose 6MB, Sephadex G-25, agarose type A and polyacrylamide-gradient gels PAA 4/30 were products of Pharmacia Fine Chemicals, Uppsala, Sweden. Poly(ethylene glycol) of mol.wt. 4000 (PEG 4000) was from AB Kebo, Stockholm, Sweden. Antisera and Partigen immunodiffusion plates, the latter for haemopexin determination, were from Behring Diagnostica, Svenska Hoechst AB, Stockholm, Sweden. *N*-Acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), puriss grade, was from Fluka AG, Buchs, Switzerland, and haematin from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade.

Poly(ethylene glycol) precipitation

In a typical experiment, 100 ml of fresh serum (pooled from four individuals) was adjusted to pH 8.0 with a few drops of 1 M-NaOH. Poly(ethylene glycol) [43 ml of a 50% (w/v) solution] was added at 4°C with stirring, which was continued for 1 h.

The suspension was centrifuged at 3300g for 15 min at 4°C. The precipitate was discarded and the supernatant was adjusted to pH 4.6 by 1M-HCl. Solid poly(ethylene glycol) (14.6g) was added to the supernatant at 4°C with stirring to give a final concentration of 25% (w/v). After 1 h, the suspension was centrifuged at 3300g for 15 min at 4°C. The precipitate was dissolved in water and the pH of the solution was adjusted to 7.5 by 1M-NaOH. The volume of the final solution was 92ml.

Chromatography on DEAE-Sepharose CL-6B

The solution containing the dissolved 15–25%-poly(ethylene glycol) precipitate was adjusted to pH 6.0 with 1M-HCl. It often became slightly turbid, in which case it was centrifuged for 10 min at 5000g. The solution was applied to a K 26/40 column (Pharmacia Fine Chemicals) containing DEAE-Sepharose CL-6B, which had been equilibrated with 0.050M-sodium acetate buffer, pH 5.2. The flow rate was 85 ml/h. The column was developed with the acetate buffer and the u.v. adsorption of the effluent monitored (see Fig. 1). This step, as well as the following ones, was carried out at room temperature (20°C).

Two sizes of columns were used, containing 77 and 155 ml of gel for 50 and 100 ml serum batches respectively. The fractions from the columns were analysed by polyacrylamide-gradient-gel electrophoresis (results not shown).

Fig. 1. Chromatography of serum fraction on DEAE-Sepharose CL-6B

A DEAE-Sepharose CL-6B column (26 mm × 145 mm; total volume 77 ml) was equilibrated with 0.050M-sodium acetate buffer, pH 5.2, at 20°C. The 15–25%-poly(ethylene glycol) fraction of human serum, adjusted to pH 6.0, was applied at a flow rate of 85 ml/h. The column was developed with the acetate buffer and 6 ml fractions were collected. The horizontal bar indicates the fractions containing haemopexin and transferrin, which were pooled.

Chromatography on wheat-germ lectin-Sepharose 6MB

The haemopexin-containing fraction from the above step was adjusted to pH 7.2 with a few drops of a saturated solution of Na_2HPO_4 . It was applied to a K 16/20 column containing wheat-germ lectin-Sepharose 6MB (9.0 ml). The gel had been equilibrated with 0.050M-sodium phosphate buffer/0.2M-NaCl/0.02% (w/v) NaN_3 , pH 7.0, and was developed at 22 ml/h with the same buffer. When the non-adsorbed material had been washed out of the column, a pulse of *N*-acetyl-D-glucosamine was applied (10 ml of buffer containing 100 mg/ml of sugar/ml). A typical chromatogram is shown in Fig. 2. The column fractions were analysed for their haemopexin content.

The eluates corresponding to the peak in Fig. 2 were pooled. The sugar was removed from the protein fraction by chromatography on a 160 ml column (26 mm × 305 mm) of Sephadex G-25 in water. The protein was analysed by polyacrylamide-gradient-gel electrophoresis and immunological methods (see below).

Protein determination

The total protein content in fractions from the different purification steps was determined as described by Lowry *et al.* (1951), with human serum

Fig. 2. Chromatography of haemopexin/transferrin mixture on wheat-germ lectin-Sepharose 6MB

A wheat-germ lectin-Sepharose 6MB column (16 mm × 45 mm; total volume 9 ml) was equilibrated at 20°C with 0.050M-sodium phosphate buffer containing 0.2M-NaCl and 0.02% (w/v) NaN_3 , pH 7.0. Crude haemopexin (Fig. 1; 140 ml) was applied to the column at 22 ml/h and 5.5 ml fractions were collected. The column was washed with equilibration buffer. The arrow indicates the application of buffer (10 ml) containing *N*-acetyl-D-glucosamine (100 mg/ml).

Fig. 3. Densitometric record of polyacrylamide-gradient-electrophoresis gel containing haemopexin samples of different haem content

Samples of pure haemopexin were incubated with an excess of haemin (23 mol/mol at pH 8.4) to increase the haem content of the protein, or with 0.5 M-acetic acid (pH 2.7) to remove haem. The samples were subjected to electrophoresis together with unmodified haemopexin on a 4–30% polyacrylamide gradient gel in 0.1 M-Tris/borate/EDTA buffer, pH 8.35, at 125 V for 16 h at 20°C. The gel was stained with Coomassie Blue R-250 and destained in 10% (v/v) acetic acid containing 30% (v/v) methanol. The scanning was performed with a Zeiss KM 3 Chromatogram Spectrophotometer. (a) Native haemopexin, (b) haemin-treated haemopexin, (c) acid-treated haemopexin. The numbers indicate the distances of migration of the sample components (in mm).

albumin as standard. Haemopexin concentrations were determined by using M-Partigen immuno-diffusion plates.

Electrophoresis

Precast 4–30% polyacrylamide gradient gels were run in 0.1 M-Tris/borate/EDTA buffer (0.0025 M-EDTA), pH 8.35, for 16 h at 125 V by using the Pharmacia Fine Chemicals gel electrophoresis apparatus GE-4 and power supply EPS 500/400. The gels were stained for 2 h in 0.25% Coomassie Blue R-250 in acetic acid/methanol/water (1:5:4, by vol). They were destained in acetic acid/methanol/water (1:3:6, by vol) by using the Pharmacia Fine Chemicals GD-4 destainer and DPS power supply (24 V, 60 min) (see Plate 1 and Fig. 3).

Immunoelectrophoresis was carried out as described by Grabar & Williams (1953) and immunodiffusion experiments were performed by using the technique of Ouchterlony (1949) (see Plates 2 and 3 respectively).

Amino acid analysis

Protein samples (0.1–1.0 mg) were hydrolysed with 6 M-HCl at 110°C for 24 h and analysed as described by Spackman *et al.* (1958) at the Biomedical Center, University of Uppsala. Proline, cysteine and methionine were measured in performic acid-oxidized samples (Hirs, 1967). Tryptophan was measured spectrophotometrically (Goodwin & Morton, 1946).

Addition and removal of haem

Purified haemopexin was treated with haemin by the technique of Latner & Emes (1975). Haematin (2 mg) was dissolved in 1.0 ml of 0.1 M-NaOH, to which was added ethylene glycol (1 ml) and 0.1 M-Tris/borate buffer, pH 8.35 (8 ml). Then 1 ml of the resulting solution was mixed with 1.0 ml of the Tris/borate buffer containing 0.75 mg of haemopexin. The mixture (final pH 8.4) was incubated for 30 min at 20°C and subjected to electrophoresis (Fig. 3). To remove haem, 2.2 mg of haemopexin was incubated for 2.5 h in 1.5 ml of 0.5 M-acetic acid (pH 2.7) and then chromatographed on a column of Sephadex G-25 (16 mm × 300 mm) in the same solvent.

Results and Discussion

Haemopexin has been difficult to purify (Muller-Eberhard & Morgan, 1975; Hrkal *et al.*, 1972; Ramsden & Louis, 1973). It has been argued that this is due to the high carbohydrate content of the protein and also to the fact that there are many serum proteins with an electrophoretic mobility similar to that of haemopexin, i.e. the β -globulins (Muller-Eberhard & Morgan, 1975). Preparations of haemopexin are often contaminated with transferrin; these two proteins have the same electrophoretic mobility at pH 8.6 (Putnam, 1975, pp. 57–131) and isoelectric

points which are very close to each other, 5.8 (Muller-Eberhard & Morgan, 1975) and 5.5–5.9 (Putnam, 1975, pp. 265–316) for haemopexin and transferrin respectively. Thus it should be difficult to prepare pure haemopexin by techniques such as ion-exchange chromatography which exploit differences between the net electric charges of the components of the sample.

However, ion-exchange chromatography is suitable for the separation of a mixture of haemopexin and transferrin from other plasma proteins. A procedure for the purification of albumin in high yield from human plasma has been developed (Curling *et al.*, 1976, 1977*a,b*). This method involves fractional precipitation by poly(ethylene glycol) and stepwise ion-exchange chromatography on DEAE-Sephacrose CL-6B. One of the fractions from the ion exchanger consists mainly of haemopexin and transferrin.

A combination of the above technique with lectin affinity chromatography was found useful for the preparation of pure haemopexin. Lectins are proteins capable of reversible binding to carbohydrates and carbohydrate-containing substances; two well-known examples are the lectins from jack bean (concanavalin A) and wheat germ, which react with mannose-like residues and *N*-acetyl-D-glucosamine respectively. Immobilized lectins have become important tools for the fractionation of polysaccharides, glycoproteins and whole cells (Liener, 1976; Gold & Balding, 1975; Hellström *et al.*, 1976). During a screening study on the use of Sepharose-bound lectins for the fractionation of human serum glycoproteins we observed (Hjorth & Vretblad, 1977) that some proteins, including haemopexin, interacted reversibly with immobilized wheat-germ lectin, whereas others, including transferrin, did not bind.

The procedure described by Curling *et al.* (1977*a*) for poly(ethylene glycol) precipitation and ion-exchange chromatography of plasma was adopted with minor modifications for 50 or 100 ml samples of serum. The precipitate obtained by using 15% poly(ethylene glycol) contained mostly immunoglobulins and lipoproteins, and was discarded. The

second precipitate, obtained when the poly(ethylene glycol) concentration was raised to 25%, contained albumin and several other proteins, notably transferrin, haemopexin, α_1 -antitrypsin and α_1 -acid glycoprotein (Plate 1).

The precipitate was dissolved and applied to a DEAE-Sephacrose CL-6B column (Fig. 1). The first peak emerging from the column consisted of haemopexin and transferrin, which have higher isoelectric points than the other components of the sample (see also Plate 1). The large peak in Fig. 1 corresponded to the albumin.

The fractions containing haemopexin and transferrin were pooled and applied to a wheat-germ lectin-Sepharose 6MB column as shown in Fig. 2. Transferrin was not retarded and gave rise to a plateau in the chromatogram. A very small amount of haemopexin leaked through the column with the last millilitres of sample, indicating that the column had become almost saturated. Desorption with *N*-acetyl-D-glucosamine caused the haemopexin to be eluted as a sharp peak: 18 mg of protein was obtained from a column containing 9 ml of gel, so the capacity of the adsorbent thus was about 2 mg of haemopexin/ml of sedimented gel.

Free sugar and salts were removed from the haemopexin by gel filtration on Sephadex G-25 in water. If the protein was not characterized immediately it was stored in freeze-dried form, in which it is very stable (Muller-Eberhard & Liem, 1974). The overall purification scheme for a 100 ml serum batch is shown in Table 1. A final yield of 37% was obtained. In one preliminary experiment on 50 ml scale we obtained a yield of about 60%.

Portions of fractions from the different purification steps were analysed by polyacrylamide-gradient-gel electrophoresis (Plate 1). This electrophoretic technique fractionates molecular species according to their size and, to some extent, their conformation. It was evident that the haemopexin had not polymerized during the purification, which has sometimes been reported (Muller-Eberhard & English, 1967), probably as a consequence of exposure of the protein to low pH. However, the shape of the haemopexin band suggested that the preparation

Table 1. *Purification of human haemopexin*

Total protein was measured by the method of Lowry *et al.* (1951) and haemopexin by using Partigen immunodiffusion plates. The wheat-germ lectin-Sepharose 6MB column had to be run twice to process all the material from the ion exchanger.

Purification step	Total volume (ml)	Total protein (mg)	Total haemopexin (mg)	Recovery of haemopexin (%)	Purification factor
Serum	100	8850	75	(100)	
Poly(ethylene glycol) precipitation	92	5750	62	83	1.3
DEAE-Sepharose CL-6B chromatography	232	223	33	44	17
Wheat-germ lectin-Sepharose 6MB chromatography	35	33	30	40	107
Sephadex G-25 gel filtration	85	29	28	37	114

EXPLANATION OF PLATE I

Electrophoresis in polyacrylamide gradient gel of fractions from different steps in haemopexin purification

The samples were run on a 4–30% polyacrylamide gradient gel in 0.1 M-Tris/borate/EDTA buffer, pH 8.35, at 125 V for 16 h at 20°C. The gel was stained with Coomassie Blue R-250 and destained in 10% (v/v) acetic acid containing 30% (v/v) methanol. (a) Normal human serum (2.5 μ l); (b) dissolved 15–25%-poly(ethylene glycol) precipitate of serum (4 μ l); (c) the contents of the first peak from (b) emerging from the DEAE-Sepharose column (18 μ l); (d) the fraction desorbed from the wheat-germ lectin-Sepharose column by *N*-acetyl-D-glucosamine (10 μ l). The positions of some known proteins are indicated: LP, β -lipoprotein; α_2 M, α_2 -macroglobulin; IgG, immunoglobulin G; DA, dimeric albumin; Cer, caeruloplasmin; Tr, transferrin; Hpx, haemopexin; A, albumin; AT, α_1 -antitrypsin; AG, α_1 -acid glycoprotein.

EXPLANATION OF PLATE 2

Immunoelectrophoresis of haemopexin and human serum against anti-haemopexin and anti-(human serum)

NaCl (0.9%, 4 μ l) containing 20 μ g of haemopexin and 4 μ l of human serum were run in 1.5% agarose at 10V/cm for 50 min at 15°C. The diffusion against antisera was left to proceed for 48 h, after which the plate was stained with 0.5% (w/v) Amido Black in 7% (v/v) acetic acid and destained in 7% acetic acid. (a) Well containing rabbit anti-(human haemopexin), (b) and (c) haemopexin, (d) well containing horse anti-(human serum), (e) human serum.

EXPLANATION OF PLATE 3

Double immunodiffusion of haemopexin against antisera to human serum proteins

The experiment was carried out in 1.5% agarose. Rabbit antisera to the following proteins were used: 1, haemopexin; 2, α_2 -macroglobulin; 3, caeruloplasmin; 4, haptoglobin; 5, α_1 -acid glycoprotein; 6, albumin; 7, transferrin; 8, α_1 -antitrypsin; 9, prealbumin; 10, immunoglobulin G; 11, immunoglobulin A; 12, immunoglobulin M.

was heterogeneous or contained an impurity. Only one band could be seen in analytical immuno-electrophoresis against anti-(human serum) and anti-haemopexin (Plate 2), indicating the presence of only one antigenic specificity in the sample. We therefore assumed that the asymmetry of the haemopexin band in gradient-gel electrophoresis arose from molecular heterogeneity.

It is known that haemopexin, when isolated from normal serum, is partially saturated with haem (Hrkál & Muller-Eberhard, 1971). The apo- and holo-proteins have been shown to behave differently in a two-dimensional isoelectric-focusing-electrophoresis system (Latner & Emes, 1975), and the possibility that our electrophoretic bands represented these molecular species was therefore investigated. One sample of haemopexin was incubated with free haem and another was incubated in acetic acid at pH 2.7, under which conditions the haem-haemopexin complex dissociates (Muller-Eberhard & Morgan, 1975). Gradient-gel electrophoresis of the reaction products together with free haemopexin (Fig. 3) indicated that the two bands in the unmodified protein did indeed represent apo-haemopexin and haem-haemopexin complex and that the protein that penetrated furthest into the gel (the minor component) was the latter. This result indicates that haem-haemopexin has a more globular conformation than apo-haemopexin. This is an interesting observation, since it is assumed that the haemopexin molecule must have a certain conformation to be able to interact with the parenchymal cells of the liver. The conformation of the molecular forms of haemopexin has been studied by spectroscopic methods (Morgan *et al.*, 1976).

As a complement to the above analytical techniques, double-immunodiffusion experiments were carried out with the haemopexin preparation against a range of antisera to human serum proteins (Plate 3). No impurities were detected by this technique.

The results of amino acid analysis of haemopexin are shown in Table 2. They are in reasonable agreement with earlier results which, however, were calculated for different values of the molecular weight (Heimbürger *et al.*, 1964; Havez *et al.*, 1967; Hrkál & Muller-Eberhard, 1971). An erroneously high value for histidine was obtained in the first analysis, probably owing to interference by haem. Incubation of a protein sample at pH 2.7, followed by gel filtration and amino acid analysis, gave a significantly lower histidine value.

The technique described in the present paper should prove useful for rapid analytical and semi-preparative-scale preparation of pure haemopexin. The whole procedure can be carried out in 2 working days. It is simultaneously a method for preparing pure transferrin, which is obtained as the unbound

Table 2. *Amino acid composition of human haemopexin*
The results were calculated for a mol.wt. of 57000 from which had been subtracted the weight of the sugar moiety, which is 11000 (value of Muller-Eberhard & Morgan, 1975). The results are means of two determinations. Proline, cysteine and methionine were determined from performic acid-oxidized samples. The value for histidine is based on one determination. Tryptophan was determined by the method of Goodwin & Morton (1946).

	Number of residues per molecule	Nearest integral number
Asx	36.2	36
Thr	21.1	21
Ser	27.3	27
Glx	36.3	36
Pro	34.1	34
Gly	38.3	38
Ala	26.8	27
Cys	12.3	12
Val	21.7	22
Met	8.0	8
Ile	8.7	9
Leu	34.8	35
Tyr	13.5	14
Phe	15.6	16
His	17.2	17
Lys	20.8	21
Arg	21.0	21
Trp	12.9	13
Total	406.6	407

fraction from the wheat-germ lectin-Sepharose. Commercial preparations of transferrin are often contaminated by haemopexin; they could easily be purified by a passage through a column containing immobilized wheat-germ lectin.

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