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Note

Rapid chromatographic separation of porcine ceruloplasmin

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Ceruloplasmin is a blue glycoprotein generally occurring in mammalian blood[1]. In spite of its oxidase activity (E.C. 1.16.3.1) it is considered a storage and transport protein for copper rather than as an enzyme[1]. Ceruloplasmin contains 6–8 copper atoms in its molecule, which are responsible for its blue colour as well as for its oxidase activities[1]. Several purification procedures for this protein are known[1–4], including different fractionations and chromatographic separations. Recently, an efficient chromatographic purification of human ceruloplasmin has been described[5].

The aim of this paper is to propose a high-performance liquid chromatographic (HPLC) procedure for the preparation of pig ceruloplasmin. The material for this purification was obtained as a by-product of the preparation of monoamine oxidase from porcine blood plasma[6].

EXPERIMENTAL

Protein preparation

Crude ceruloplasmin was prepared from the dialysed fraction of porcine blood plasma (30–60% saturation with ammonium sulphate) by chromatography on a DEAE-cellulose column[6]. The column was equilibrated and washed with 30 m*M* sodium phosphate buffer pH 7. The fractions containing monoamine oxidase were eluted specifically using 10 m*M* benzylamine in the same buffer and another protein fraction (containing ceruloplasmin) was obtained by washing the column with 0.1 *M* sodium phosphate buffer pH 7[6]. The crude ceruloplasmin prepared in this way was a generous gift from Dr. L. Macholán. It was stored at −20°C, thawed immediately before use and concentrated with an ultrafiltration cell (Amicon, Danvers, MA, U.S.A.) with a XM-50 membrane.

The following HPLC separations were carried out at room temperature, the fractions eluted from the column being kept at 4°C. Gel permeation chromatography (GPC) was performed using a preparative UltroPac TSK 3000 SWG (600 mm × 21.5 mm I.D., 13 μm) from LKB (Bromma, Sweden) equipped with a precolumn (LKB). A Mono Q column (HR 5/5, 50 mm × 5 mm I.D., 10 μm) from Pharmacia (Uppsala, Sweden) served for the final high-performance anion-exchange chromatography. The columns were attached to two P-500 pumps and a GP-250 gradient programmer (Pharmacia). The separation were evaluated by means of an UV-1 mon-

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itor (λ = 280 nm), a REC-482 recorder and a FRAC-100 fraction collector (Pharmacia). The samples were injected with a V-7 valve (equipped with a 10-ml Superloop) from Pharmacia. The purified ceruloplasmin was concentrated as mentioned above.

Protein analysis

The ceruloplasmin and protein contents were measured spectrophotometrically at 610 and 280 nm, respectively, in a Cary 118 instrument (Varian, Palo Alto, CA, U.S.A.). The absorption coefficients were taken rom ref. 2. The oxidase activity of ceruloplasmin was assayed at pH 5.5 and 30°C with 10 m*M* *p*-phenylenediamine as the substrate[6]. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out as described[7].

The chemicals were from Serva (Heidelberg, F.R.G.) and Lachema (Brno, Czechoslovakia).

RESULTS AND DISCUSSION

The starting material (containing *ca.* 16% of ceruloplasmin, Table I) obtained as a by-product of the isolation of porcine monoamine oxidase proved to be suitable for the preparation of pure ceruloplasmin. The first HPLC step, *i.e.*, GPC on a preparative TSK 3000 SWG column, resulted in a good separation of contaminating proteins with molecular weights exceeding that of ceruloplasmin (*ca.* 140 000[1]), whereas the separation of smaller proteins was incomplete (Fig. 1 and Table I). The extent of purification of ceruloplasmin was three- to four-fold in this step, the recovery being better than 80% (Table I). The elution was carried out with a 20 m*M* sodium phosphate buffer pH 6.9. The use of more concentrated buffers brought about a negligible improvement of the separation efficiency, and the weaker buffer was preferred since the eluted sample could be used directly in the next purification step.

Fig. 2 shows that the remaining contaminants were removed completely by high-performance anion-exchange chromatography on a Mono Q column. The recovery of ceruloplasmin approached 90% when elution with increasing sodium phos-

TABLE I

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PREPARATION OF PORCINE CERULOPLASMIN

The data are averages calculated from three preparations. The protein and ceruloplasmin contents were determined from the absorbances at 280 and 610 nm, respectively.

Fraction	*Protein (mg)*	*Ceruloplasmin (mg)*	*Purity (%)*	*Yield in each step (%)*
Crude preparation*	130	21	16	–
After TSK 3000 SWG chromatography	33	18	54	85
After Mono Q chromatography**	16	16	100	89

* The starting material was obtained from a DEAE-cellulose column by elution with 0.1 *M* sodium phosphate buffer (pH 7).

** The separation proceeded in three consecutive steps.

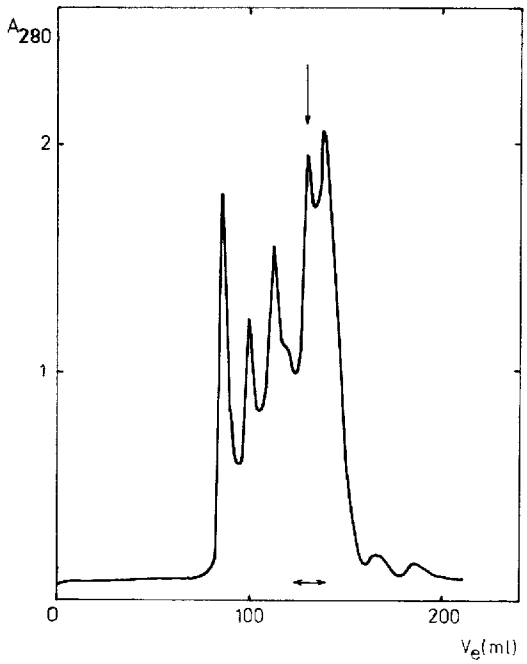

Fig. 1. Chromatography of crude ceruloplasmin on a UltroPac TSK 3000 SWG column. Mobile phase: 20 m*M* sodium phosphate buffer (pH 6.9). V_e = elution volume; ———, absorbance at 280 nm (A_{280}); the arrow corresponds to ceruloplasmin (A_{610}); ↔, the fractions collected for the next step. Approximately 130 mg of protein were injected.

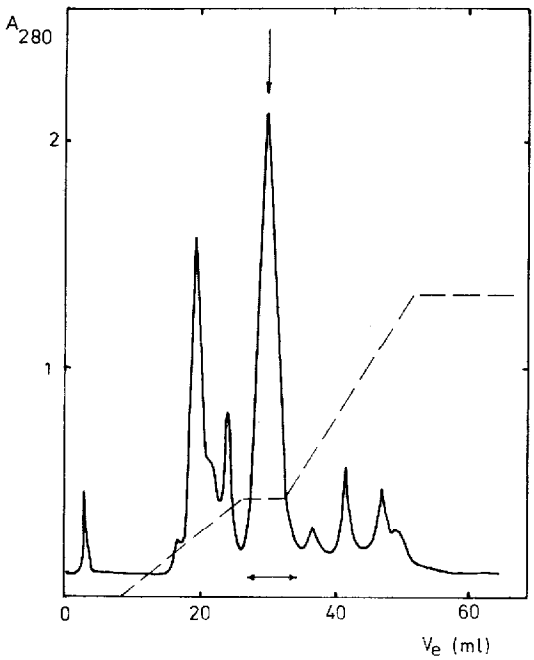

Fig. 2. Chromatography of ceruloplasmin on a Mono Q column. Buffers: A = 20 m*M* sodium phosphate (pH 6.9); B = 0.3 *M* sodium phosphate (pH 6.9). Approximately 11 mg of protein were injected. Other details as in Fig. 1.

phate concentrations (pH 6.9) was used. The results obtained with the other concentration gradients tested, *i.e.*, sodium chloride, lithium chloride and sodium acetate were inferior. The small amount of proteins eluted at higher sodium phosphate concentrations than used for ceruloplasmin (Fig. 2) is due to the fact that the starting material was obtained from an anion-exchange column eluted with 0.1 *M* sodium phosphate. When the GPC step (Fig. 1) was omitted and the starting material was applied directly to a Mono Q column (after dia-filtration) the results were poorer —the proteins eluted prior to and closely after ceruloplasmin were more abundant. The purity of the resulting ceruloplasmin was only 70% in this case. Gel permeation chromatography largely removes these proteins, moreover, it replaces the dia-filtration step.

The purity of the obtained ceruloplasmin approached 100% (on the basis of the ratio A_{610}/A_{280}[2]), its yield being *ca.* 75%. The specific oxidase activity of the purified ceruloplasmin (nearly 20 nkat/mg protein) corresponded to that given in ref. 6. Re-chromatography the product on an analytical UltroPac TSK 3000 SW column (LKB), on a Superose 12 column (Pharmacia) or on a Mono Q column (after dia-filtration) yielded only one protein peak. The same result was also obtained with SDS-PAGE. The HPLC separation presented here is suitable for a quick purification of relatively small quantities of homogeneous porcine ceruloplasmin (Table I). Larger amounts of ceruloplasmin can be obtained by repeated GPC and with larger high-performance anion-exchange columns, *e.g.*, Mono Q HR 10/10, 100 mm × 10 mm I.D.). One of the advantages of the proposed method is that it uses waste material which remains from the preparation of another protein. This starting material can be stored for several years at −20°C without appreciable decrease in the ceruloplasmin content. The whole procedure (including the concentration of the initial material and that of the final product) takes less than 5 h, and the presence of sodium phosphate in the final preparation acts as a stabilizer. In principle, the described procedure is suitable for the preparation of ceruloplasmin from other animals.

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