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Isolation and partial characterization of molecular forms of ceruloplasmin from human bile

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Highly purified ceruloplasmin (CP) was isolated from human bile using affinity chromatography. Biliary CP is represented by two molecular species. One of those is identical to oxidase CP from normal human serum while the other is analogous to oxidase-lacking CP specific for the serum of the carriers of Wilson's mutation with respect to immunological specificity, electrophoretical mobility and molecular mass of the large fragments from spontaneous proteolysis.

Ceruloplasmin, Wilson's disaese, Human bile

1. INTRODUCTION

A CP-like protein has been detected recently in human bile [1]. Since its content in the bile correlated directly with the amount of copper consumed with the food, a suggestion was made that this protein is involved in the copper excretion via bile and in the prevention of copper re-absorption from the intestine. This protein was not detected immunologically in the bile of patients with Wilson's disease[1] [1]. On the other hand, a protein immunologically related to biliary CP was found in the sera of homo- and heterozygous carriers of Wilson's disease gene. This oxidase-negative species of CP appeared to be specific for the carriers of Wilson's mutation and, therefore, was referred to as Wilsonian CP [2].

Hence, purification of CP from normal human bile and its comparison with the serum counterpart from HLD patients may contribute to a better understanding of both the physiological role of CP in copper metabolism and the molecular mechanism of its inherited defect in Wilson's disease.

2. MATERIALS AND METHODS

The bile obtained via biliary duct drainage in patients with gallstones after cholecystectomy served as the source of biliary CP. The commercial CP preparation was obtained from Pasteur Institute of Epidemiology and Microbiology, St. Petersburg.

Abbreviations: CP, ceruloplasmin; HLD, hepatolenticular degeneration (Wilson's disease); PAAG, polyacrylamide gel; SDS, sodium dodecyl sulphate; PEG, polyethylene glycol.

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Affinity sorbent for CP was synthesized using cyanogen bromide-activated Sepharose 4B [3]. Hexamethylene diamine and succinic anhydride served as spacers, while *p*-phenylene diamine was the CP-specific ligand.

Isolation of homogeneous Wilsonian CP and preparation of monospecific antibodies against this protein were described elsewhere [2,4].

Electrophoresis under native and denaturing conditions, immunoelectrophoresis and immunoblotting were carried out according to the routine procedures [5–8].

3. RESULTS AND DISCUSSION

The affinity chromatography on *p*-phenylene diamine-Sepharose 4B allowed the single-stage isolation of both normal and Wilsonian CPs from human sera in a high yield [4]. However, our attempts to obtain CP preparations from the whole bile using this single-stage method failed. That was due to high affinity of bile pigments to amino groups of the sorbent, which interfered with CP sorption. The bulk of bile pigments was removed by precipitation of proteins (including CP) in 25% PEG 6000 for 12 h at 4°C (the extent of CP precipitation was controlled using rocket immuno-electrophoresis). Nevertheless, a minor admixture of of protein-bound bile pigments left after PEG precipitation prevented the direct isolation of CP from this material using affinity chromatography.

The effective removal of bile pigments was achieved by 25% PEG 6000 precipitation followed by chromatography of precipitated material on Sephadex G-100. Fig. 1 shows that biliary proteins were eluted from Sephadex G-100 in three peaks. The elution volume of the first peak (high molecular mass proteins) was the same

[1]Autosomal recessive defect of the excretion of excessive copper from the body.

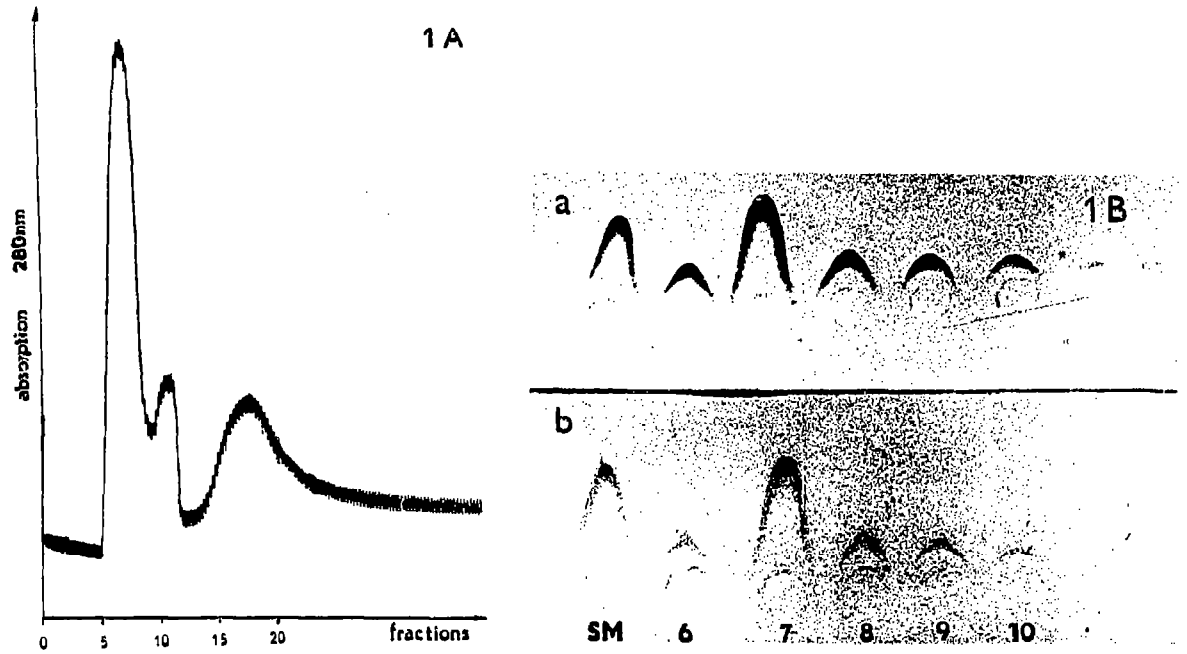

Fig. 1. Gel-filtration of biliary proteins precipitated with PEG. 50 ml of bile were mixed with 15 g of PEG 6000. The precipitate was collected by centrifugation, washed with 25% PEG and dissolved in 10 ml of 0.05 M sodium acetate buffer, pH 5.5. The suspension was cleared by centrifugation and applied to Sephadex G-100 column (2 × 50 cm), and equilibrated with the same buffer. Fraction volume was 7 ml. (A) Elution profile. (B) Rocket immunoelectrophoresis of the starting material (SM), and of the fractions of eluate (6–10). Immunoelectrophoresis was carried out in 1% agarose gels containing 0.1 mg/ml IgG from immune sera. (a) Antibodies to normal oxidase-positive CP, *o*-dianizidine staining. (b) Monospecific antibodies to Wilsonian CP, Coumassie R-250 staining.

as that of purified preparation of human serum CP used as a marker to calibrate the column.

The immunological estimations showed that both normal CP and Wilsonian CP were eluted in the first peak (see Fig. 1B). The CP-containing fractions of this peak were pooled, re-precipitated by 25% PEG, pelleted in a centrifuge, dissolved and fractionated by ion exchange chromatography on a DEAE-Sepharose column

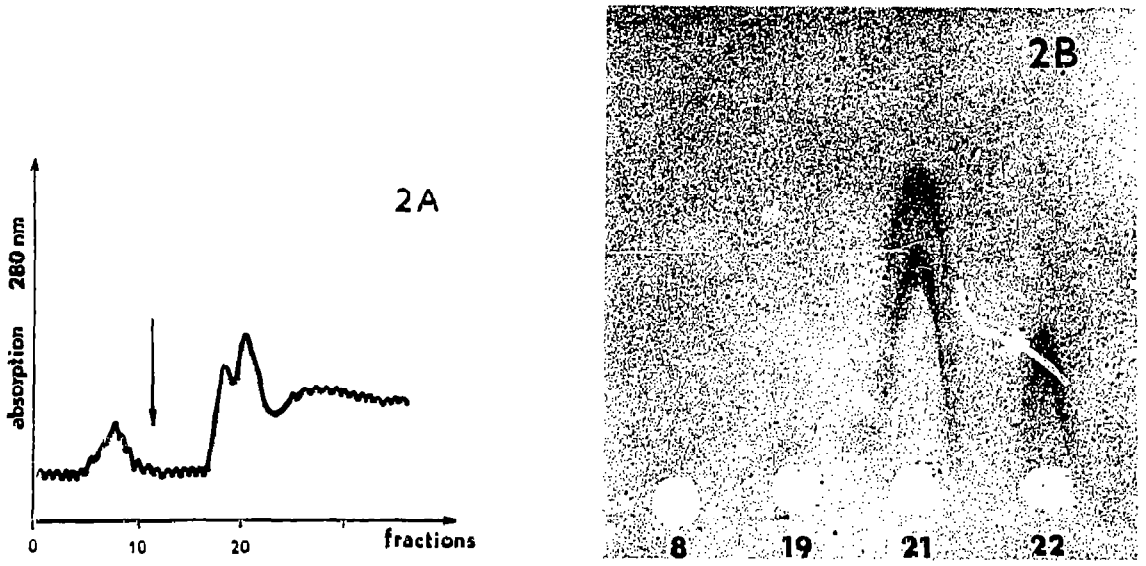

Fig. 2. Ion-exchange chromatography of CP-containing fraction of biliary proteins obtained upon gel-filtration. CP-containing fractions eluted from Sephadex G-100 column were pooled and applied to a 50-ml DEAE-Sepharose column equilibrated with 0.05 M sodium acetate buffer, pH 5.5. Fraction volume was 7 ml. (A) Elution profile. The first peak (fractions 6–10) corresponds to unbound proteins (eluted with the equilibrating buffer). The arrow indicates the switch to the eluting buffer (0.3 M sodium chloride, 0.05 M sodium acetate buffer, pH 5.5). (B) Rocket immunoelectrophoresis of the eluate fractions (8, 19, 21 and 22) in 1% agarose gel containing antibodies to oxidase CP and monospecific antibodies to Wilsonian CP (100 μg/ml of each). The gel was stained with *o*-dianizidine and then with Coomassie R-250. *o*-Dianizidine stained only internal rockets.

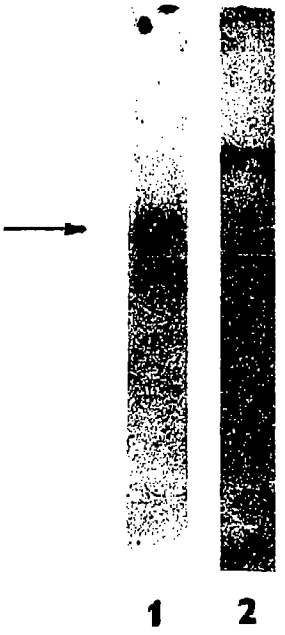

Fig. 3. Non-denaturing PAAG electrophoresis-immunoblotting of biliary CP. Purified biliary CP was fractionated by electrophoresis in 7.5% PAAG, pH 8.8, under non-denaturing conditions. The protein was transferred onto nitrocellulose filter by electroelution and analyzed by immunoblotting with antibodies to normal CP (1) and monospecific antibodies to Wilsonian CP (2). The bands containing bound antibodies were detected with [^{125}I]protein A, followed by radioautography. The arrow indicates the position of the band of CP isolated from donor serum.

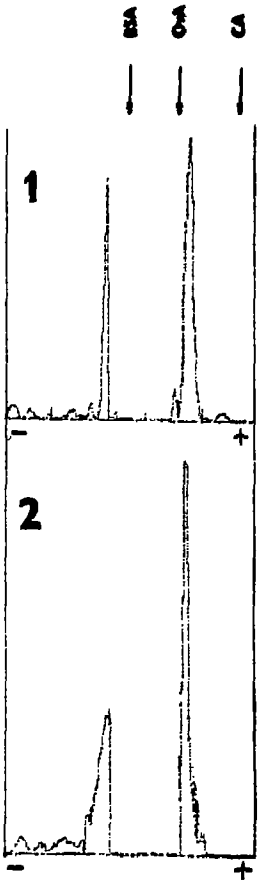

Fig. 4. Denaturing gel-electrophoresis of Wilsonian CP from HLD serum and of biliary CP which interacts with monospecific antibodies to Wilsonian CP. CP preparations from HLD serum (1) and from human bile (2) were subjected to electrophoresis under non-denaturing conditions in 7.5% PAAG. Gel strips corresponding to the location of oxidase-negative CP species were cut out, homogenized and re-electrophoresed in denaturing 7.5% PAAG-SDS, according to Laemmli [7]. Protein bands were electroeluted onto nitrocellulose filter and incubated with monospecific antibodies against Wilsonian CP. The bands containing bound antibodies were detected, using peroxidase-conjugated protein A. The intensity of peroxidase-stained bands was recorded in laser densitometer (Biomed. International, USA). Arrows indicate the positions of molecular mass markers: bovine serum albumin (BSA) = 66 kDa; ovalbumin (OvA) = 45 kDa; carbonic anhydrase (CA) = 29 kDa.

which, as had been shown earlier [4], retained normal CP, but did not adsorb Wilsonian CP. The immunological assay of eluate fractions showed that biliary CP was fully retained by DEAE-Sepharose (Fig. 2).

Subsequent affinity chromatography was carried out using the CP-specific sorbent. The CP-containing fractions eluted from ion exchange column were pooled, dialyzed against 0.05 M sodium acetate buffer, pH 5.5, and applied to the column with the affinity sorbent equilibrated with the same buffer. After the column was washed with equilibrating buffer, the retained proteins were eluted with 0.3 M sodium chloride. As was evident from immunoelectrophoretic data (not shown), the protein fractions washed out with equilibrating buffer contained no immunoreactive CP; both molecular forms of biliary CP having been retained on the sorbent and eluted with 0.3 M sodium chloride.

Electrophoresis of these fractions under non-denaturing conditions showed that CP migrated as two bands stained with Coomassie R-250. One of those could be stained also with *o*-dianizidine, a specific chromogenic substrate for CP oxidase activity, and corresponded to the normal serum CP in its migration rate. This fraction was recognized by antibodies to normal CP when immunoblotting was applied (Fig. 3). The other band was not stained with *o*-dianizidine and interacted only with monospecific antibodies to Wilsonian CP.

Two similar protein bands were also detected earlier, when CP preparations from HLD sera were fractionated electrophoretically under non-denaturing conditions [2,4]. One (fast) band was stained with *o*-dianizidine and had the mobility of CP isolated from healthy donor blood. The other (slow) band, lacked oxidase activity, but interacted with antibodies to CP. Isolation of this CP (Wilsonian CP), its characterization and preparation of monospecific antibodies have been described in our previous paper [2].

Biliary CP recognized by the latter antibodies and oxidase-negative CP isolated from the serum of HLD patients were compared using PAAG-SDS electropho-

resis. It was shown (Fig. 4) that the biliary CP interacting with monospecific antibodies against Wilsonian CP was represented by two polypeptides (80 and 40 kDa) identical in their molecular mass to those of Wilsonian CP.

These data allow one to conclude that normal human bile contains at least two molecular forms of CP. With respect to their oxidase activity, antigenic properties, electrophoretic mobility, one of them is identical to the normal serum CP, while the other is similar to Wilsonian CP.

Now, the question arises how these CP forms appear in the bile. It has been shown earlier that the membrane fraction of hepatocytes from normal human liver contains polypeptides related antigenically to Wilsonian CP [9]. Besides, we showed in the model experiments that rat liver cells synthesize a molecular form of CP which is not secreted into the blood stream [10]. These facts alongside with the data on the role of liver lysosomes in the copper excretion [11] suggest that biliary CP species devoid of oxidase activity is involved in the binding of copper taken up by hepatocytes from the blood. The copper bound to this protein is likely to be either re-incorporated into a newly synthesized secretory CP, or as a part of the oxidase-negative CP is excreted via lysosomes into the bile.

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REFERENCES

[1] Iyengar, V., Brewer, G.F., Dick, R.D. and Owyang, C. (1988) J. Lab. Clin. Med. 111, 267–274.
[2] Puchkova, L.V., Verbina, I.A., Denezhkina, V.V., Vakharlovskii, V.G., Voitsekhovskii, B.L., Gaitskhoki, V.S. and Neifakh, S.A. (1990) Biomed. Sci. 1, 460–466.
[3] Cuatrecasas, P. (1972) Adv. Enzymol. 36, 29–89.
[4] Verbina, I.A. and Puchkova, L.V. (1985) Doklady Akademii Nauk SSSR 283, 478–480 (in Russian).
[5] Kroll, J. (1973) in: A Manual of Quantitative Immunoelectrophoresis (N.H. Axelsen, J. Kroll and B. Weelke eds.) pp. 108–115, Universitetsforlaget Oslo.
[6] Davis, D.J. (1964), Ann. NY Acad. Sci. 121, 404–427.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. (1984) Gen. Anal. Techn. 1, 3–8.
[9] Puchkova, L.V., Verbina, I.A., Denezhkina, V.V., Gaitskhoki, V.S. and Neifakh, S.A. (1991) Biopolimery i Kletka 7, 24–30 (in Russian).
[10] Gaitskhoki, V.S., Voronina, O.V., Denezhkina, V.V., Pliss, M.G., Puchkova, L.V., Schwartzman, A.L. and Neifakh, S.A. (1990) Biokhimia 55, 927–937 (in Russian).
[11] Gross, J.B., Myers, B.M., Kost, I.J., Kuntz, S.M. and La Russo, N.F. (1989) J. Clin. Invest. 1, 30–39.