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Young camel ceruloplasmin: Purification and partial characterization

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Key words

Dromedary - Young animal - Ferroxidase - Purification - Molecular weight - Chromatography - Electrophoresis.

Summary

Ceruloplasmin of a young camel (six months old) was isolated and purified using the single-step chromatography on amino ethyl-derivatized sepharose. The molecular mass of the protein, as estimated by non-reducing SDS-electrophoresis, was approximately 130,000 Da. The electrophoretic mobility of the young camel ceruloplasmin was slightly higher as compared to the human protein suggesting that the protein is compact and more acid. The copper content was estimated at 5.8 ± 0.3 atoms per molecule. The spectrocopic features included an absorption maximum at 610 nm, which could be attributed to type 1 copper. The EPR spectrum was completely devoid of any typical signal of type 2 copper. p-phenylendiamine oxidase properties of the young camel ceruloplasmin were determined: Km = 0.42 µM NADH/mn/mg Cp and Vmax = 0.93. Optimum pH for the activity was 5.7.

■ INTRODUCTION

Ceruloplasmin (Cp) is a blue multi-copper oxidase, which is found in the plasma of vertebrate species. The protein is synthesized in the liver as a single polypeptide chain and secreted into the plasma with six atoms of copper bound per molecule (11). Cp is a multifunctional glycoprotein and plays different roles in a number of biological processes including tissue angiogenesis, copper transport (19), iron metabolism (13), and antioxidant defense (9). Cp is present in the γ globulin fraction of vertebrate plasma and 95% of plasma copper is bound to it (7). Cp contains 5-7 copper atoms per molecule, which can be separated into three types, based on their spectroscopical attributes (8). Type 1 or "blue" copper is responsible for the unusually strong electronic absorption around 600 nm and is paramagnetic. Type 2 "non blue" copper is essentially silent in the optical spectrum, but contributes to the electronic paramagnetic resonance (EPR) spectrum with a linshape quite typical of regularly coordinated tetragonal complexes. Type 3 copper consists of a pair of metal ions, antiferro-magnetically-coupled to give an EPR-silent species. It absorbs in the near UV region of the electronic spectrum, giving a shoulder around 330 nm (2, 14, 15). Most studies were conducted on human ceruloplasmin. The protein has also been successfully isolated from the plasma of other mammals (18), marine mammals (1), birds (7) and reptiles (16).

In camel, Cp oxidase activity seems to be maintained when copper levels in plasma decrease drastically. The correlation coefficient between plasma copper concentration and ceruloplasmin activity in camel is improved when the exponential regression is calculated (10). This suggests that biochemical properties of camel Cp could be slightly different from those in other domestic species. Purification of camel Cp would be helpful to understand if this difference could be attributed to a specific structure of camel Cp. The objective of the work reported here was Cp purification from a new source; Cp from the young camel was isolated and partially characterized.

■ MATERIALS AND METHODS

Chemicals

All chemicals were reagent grade and were used without further purification. Sepharose 4B was purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden.

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Sample collection

Plasma from camel was collected at Settat slaughterhouse from a young camel (male) 6-8 months old. The blood was centrifuged immediately and the plasma was harvested. The plasma was kept frozen at -20°C until use.

Preparation of derivatized Sepharose

Cp purification was performed in a single chromatographic step on a Sepharose derivative obtained by reaction of Sepharose with chloroethylamine according to the published procedure (7). Sepharose 4B (300 ml) previously treated at 70°C for 2 h with 5 N NaOH (100 ml) and epichlorydrin (25 ml), and exhaustively washed, were suspended in 10 N NaOH and treated with 100% chloryethylamine (140 ml) for 2 h at 70°C. The resulting derivatized Sepharose was equilibrated at pH 7.4 with 3 mM phosphate buffer.

Isolation of camel ceruloplasmin

Young camel plasma was diluted with 20 mM 6-aminohexanoic acid to prevent proteolysis until conductibility was as low as 7 ms at 18°C. After adjusting to pH 7.4 by addition of small aliquots of H_3PO_4, the plasma was loaded on a column of derivatized Sepharose. An intense blue band appeared on the top of the column. The resin was washed by 50 mM (300 ml), 80 mM (200 ml), 100 mM (100 ml) and 150 mM (100 ml) phosphate buffer pH 7.4. Ceruloplasmin was eluted with 200 mM phosphate buffer and fractions with A_{610}/A_{280} near 0.04 were pooled and concentrated on YM 100 membranes in Amicon cells. The whole purification procedure was carried out at 4°C.

Electrophoretic analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ceruloplasmin was performed according to Weber and Osborn (22). The following proteins of known molecular weight were used: Myosin rabbit muscle 205,000; ß-galacosidase *E. coli* 116,000; phosphorylase b, rabbit muscle 97,000; fructose 6 phosphatase 84,000; bovine serum albumin 66,000; glutamic deshydrogenase; bovine liver 55,000; ovalbumin; chicken egg 45,000 and glyceraldehyde 3PD; rabbit muscle 36,000. To detect oxidase activity, gels were incubated with o-dianisidine 0.5 mg/ml in 50 mM acetate buffer as described by Schosinski *et al.* (21).

Determination of protein concentrations

Protein concentration was determined according to Brown *et al.* (3) using the bicinchonic acid.

Copper content measurements

Total copper (Cu) in purified camel ceruloplasmin was determined by the chemical method of Brumbey and Massey (4) using biquinoline. The stoichiometry of copper atoms/protein molecule was estimated based on protein concentrations.

p-phenylendiamine(pPD) oxidase activity

Enzyme activity as pPD oxidase was assayed as described by Lovstad and Frieden (13) on the basis of NADH consumption by the first oxidation product of pPD. The mixture contained 0.25 mM of DTPA, 0.25 mM NADH and 1.3 µM ceruloplasmin in 0.1 M phosphate buffer at pH 6.3.

Spectroscopic measurements

Optical spectra were recorded either on a Perkin-Elmer 330 spectrophotometer equipped with Haake model G temperature controller unit. The EPR spectrum was measured on a Varian E-9 spectrometer operating at 9.15 GHz equipped with a stellar variable temperature unit and interfaced to a stellar Prometheus data system for computer analysis and handling of the spectra.

■ RESULTS AND DISCUSSION

Chromatographic elution

The protein was eluted as a single, symmetric peak with 200 mM phosphate buffer at pH 7.4. Only fractions with A_{610}/A_{280} near 0.04 were collected and concentrated on Amicon cells.

Molecular properties

Purified young camel ceruloplasmin was examined by SDS-PAGE in denaturing conditions, both in the presence and absence of reducing agents. Polyacrylamide-gel electrophoresis of purified camel Cp stained for both activity and protein is shown in figure 1. A single protein band, which accounted for over 95% total protein, corresponded to the activity zone indicating a homogeneous preparation. The molecular weight of the camel protein was estimated as 130,000 ± 4000 Da in a separate set of experiments by comparison of the relative mobility on SDS-PAGE of camel Cp with a series of standard molecular weight markers (figure 2). Human Cp, without heat treatment at 100°C for 3 min prior to SDS-PAGE, retains a native, oxidase-active conformation and migrates as a doublet, due to the presence of two forms of the protein differing in carbohydrate content (20). When analyzed in these conditions, purified camel Cp revealed a single component by both protein and oxidase activity-stained gels (figure 1). This behavior was also found in sheep, cattle and dolphin Cp (1), thus suggesting a similar native shape for all mammalian ceruloplasmins. The electrophoretic mobility of camel Cp was slightly higher than that of human Cp. This might be attributed to a difference in protein conformation. The conformation of camel Cp might be more compact and more acid than human protein.

Spectroscopic properties of camel ceruloplasmin

Chemical analysis of the copper content in young camel Cp gave a stoichiometry of 5.8 ± 0.3 Cu atoms/protein molecule, in line with the generally accepted values (5-6 Cu atoms) for ceruloplasmin (19). This result is comparable to those reported for other ceruloplasmins purified with the same single-step procedure (7, 8, 16).

Figure 3 reports the absorption spectrum of camel Cp in the visible and near UV-visible region. The optical spectrum of young camel Cp was similar to those found in other mammals. Camel Cp showed the typical absorption band centered at 610 nm due to type 1 or “blue” copper, and the shoulder at 330 nm assigned to type 3 copper. These observations are identical to those found for human (6), sheep (5) and dolphin ceruloplasmins (1), but different from those found for chicken (7) and reptilian ceruloplasmins, in which the blue band is centered at 603 nm (16).

Figure 4 shows the EPR spectrum of purified camel Cp. The EPR spectrum was completely devoid of any typical signal of type 2 copper. The spectrum obtained remained similar and identical to that reported in other studied ceruloplasmins (1, 2, 16).

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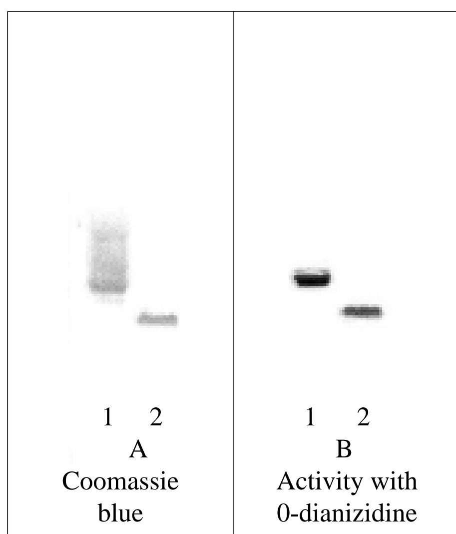

Figure 1: *PAGE-electrophoresis of young camel ceruloplasmin. Cp stained for protein (A) and for activity (B). 1: Human Cp; 2: Young camel Cp.*

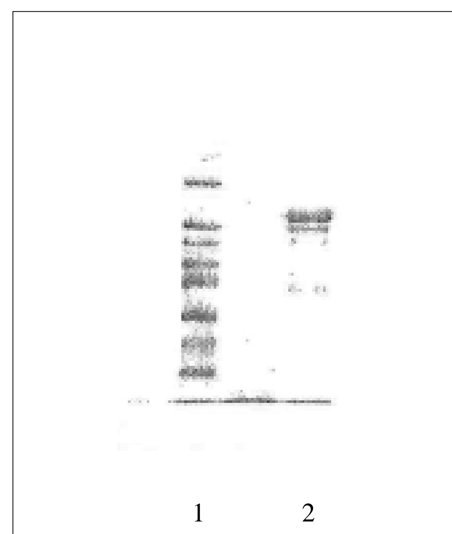

Figure 2: *SDS-PAGE electrophoresis of young camel ceruloplasmin. 1: Molecular markers; 2: Young camel Cp.*

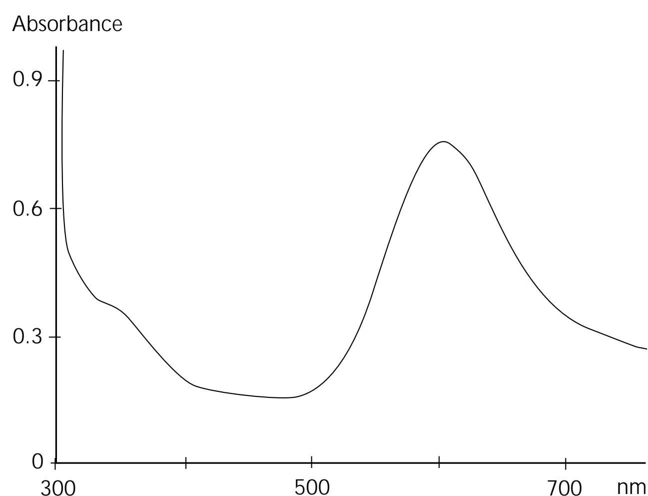

Figure 3: *Optical spectrum of young camel ceruloplasmin in 0.1 M phosphate buffer.*

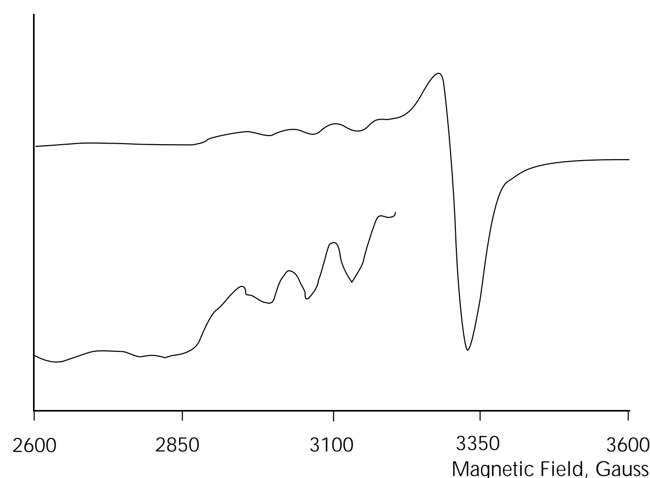

Figure 4: *EPR spectrum of young camel ceruloplasmin in 0.1 M phosphate buffer.*

Enzyme properties

pH optimum

pPD oxidase activity of young camel Cp was carried out in different pH. Optimum pH for the activity was 5.7. This pH was relatively weak compared to sheep and human Cp with an optimum pH of 6.3 and 6.4, respectively (5, 17).

Kinetic parameters

Turnover kinetic parameters of camel ceruloplasmin for p-phenylendiamine oxidase activity were estimated as follows: Km = 0.42 µM (NADH)/mn/mg ceruloplasmin and Vmax = 0.93. Young camel Cp showed a lower affinity to the substrate in comparison with turtle (0.019), chicken (0.085) and sheep (0.22) Cp (16).

■ CONCLUSION

Ceruloplasmin was isolated to purity from the young camel. There were no gross differences in the chemicophysical parameters of camel ceruloplasmin as compared to other mammal ceruloplasmin. The protein retained the same spectroscopic parameters, indicative of an essentially invariant molecular architecture. Preparations of camel ceruloplasmin showed that the protein was very homogenous.

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Résumé

Essamadi A.K., Bengoumi M., Zaoui D., Faye B., Bellenchi G.C., Musci G., Calabrese L. Céruloplasmine du chamelon : purification et caractérisation partielle

La céruloplasmine d'un chamelon âgé de six mois a été isolée et purifiée en une seule étape, utilisant une chromatographie sur Sépharose activée par de la chloroéthylamine. La masse moléculaire de la protéine a été déterminée par électrophorèse avec SDS et a été estimée à 130 000 Da. La protéine possède une mobilité électrophorétique légèrement supérieure à celle de l'homme, ce qui suggère que la céruloplasmine du chamelon est compacte et plus acide. Le nombre d'atomes de cuivre par molécule de céruloplasmine a été de 5,8 ± 0,3. Le spectre optique de la céruloplasmine du chamelon a montré une absorption maximale à 610 nm attribuée au cuivre de type 1. Le spectre EPR a été totalement dépourvu d'un signal correspondant au cuivre de type 2. Les paramètres cinétiques de l'activité oxidasique, utilisant la p-phénylendiamine comme substrat, ont été déterminés : Km = 0,42 µM NADH/mn/mg céruloplasmine et Vmax = 0,93. Le pH optimal de l'activité a été de 5,7.

Mots-clés : Dromadaire - Jeune animal - Ferroxydase - Purification - Poids moléculaire - Chromatographie - Electrophorèse.

Resumen

Essamadi A.K., Bengoumi M., Zaoui D., Faye B., Bellenchi G.C., Musci G., Calabrese L. Ceruloplasmina en el camello joven: purificación y caracterización parcial

Se aisló y se purificó la ceruloplasmina de un camello joven (seis meses de edad), mediante cromatografía única en sefarosa amino etil derivada. La masa molecular de la proteína, estimada mediante electroforesis SDS no reductora, fue aproximadamente de 130 000 Da. La movilidad electroforética de la ceruloplasmina del camello joven es ligeramente superior si comparada con la proteína humana, sugiriendo que la proteína es compacta y más ácida. El contenido de cobre fue estimado en 5,8 ± 0,3 átomos por molécula. Las cualidades espectroscópicas incluyeron una absorción máxima a 610 nm, la que podría atribuirse al tipo cobre 1. El espectro EPR fue completamente falto de señales típicas del tipo cobre 2. Los parámetros cinéticos de la ceruloplasmina del camello joven para la actividad específica de la oxidasa fenilendiamina-p, se determinaron como Km = 0,42 µM NADH/mn/mg Cp y Vmax = 0,93. El pH ideal para la actividad fue de 5,7.

Palabras clave: Dromedario - Animal joven - Ferroxidasa - Purificación - Peso molecular - Cromatografía - Electroforesis.