

A Novel and One-step Purification of Human Ceruloplasmin by Acharan Sulfate Affinity Chromatography

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Human ceruloplasmin, a copper binding α_2 -glycoprotein, was purified by a single-step procedure using acharan sulfate affinity chromatography. Acharan sulfate was immobilized to amine-functionalized agarose matrix through carboxylic acids. Ceruloplasmin in human plasma was obtained from 0.4 M NaCl salt elution and characterized by SDS-PAGE (132 and 125 kDa), isoelectric focusing (pI 4.6), Western blotting, and MALDI-TOF-MS peptide mass fingerprinting. Ceruloplasmin was purified 106 fold with a specific oxidase activity of 0.53 U/mg protein.

Key words: Acharan sulfate, Affinity chromatography, Copper binding α_2 -glycoprotein, Human ceruloplasmin, Protein purification

INTRODUCTION

Acharan sulfate (AS) was first isolated and characterized from the body of the African giant snail *Achatina fulica* Bowdich in 1996 (Kim et al., 1996). It has the repeating disaccharide unit of $\rightarrow 4$ - α -D-GlcNpAc (1 \rightarrow 4)- α -L-IdoAp2S(1 \rightarrow that is a novel structure, related but significantly different from heparin and heparan sulfate (GlcNpAc, *N*-acetylglucosamine; IdoAp2S, 2-*O*-sulfoinduronic acid). The disaccharide unit of AS is shown in Fig. 1. Our laboratory has been extensively involved in studies on AS, which exhibits interesting biological activities such as anti-angiogenic, anti-tumor activities (Lee et al., 2003) and anti-thrombotic activities *in vivo* (Li et al., 2004).

Ceruloplasmin (Cp) is a blue and copper binding α_2 -glycoprotein that contains six copper atoms per molecule and accounts for more than 95% of the total circulating copper in healthy adults. Cp belongs to the family of multi-copper oxidases towards a number of substrates, including ferrous ion, and aromatic dia-

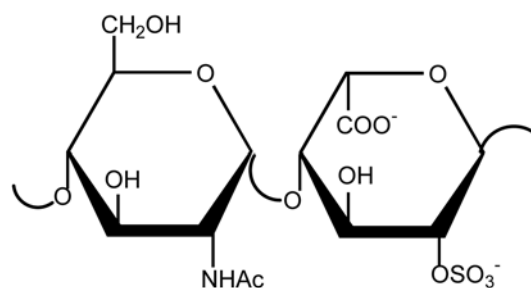


Fig. 1. Disaccharide repeating unit of AS: $\rightarrow 4$ - α -D-GlcNpAc (1 \rightarrow 4)- α -L-IdoAp2S(1 \rightarrow).

mines (Bielli and Calabrese, 2002). The mutation of Cp gene, termed aceruloplasminemia (for example, Wilson disease), results in impaired Cp production and altered iron metabolism (Harris et al., 1995). The patients with Wilson disease fail in the circulation of copper ion as Cp, which causes the deposition of copper in the liver, cornea, kidney, bones, and parathyroids. Size exclusion chromatography – inductively coupled plasma mass spectrometry (SEC-ICPMS) was employed to determine Cp from human plasma based on the ratio of ⁶³Cu and ⁶⁵Cu (Lopez-Avila et al., 2006).

The purification of Cp from various plasma sources (human, camel, bovine, horse, rat, chicken, goose, and rat) was described in the literatures (Linder and Moor, 1977; Essamadi et al., 2002; Calabrese et al., 1981;

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Calabrese et al., 1988; Hilewicz-Grabska et al., 1988; Okumura et al., 1991; Ehrenwald and Fox 1994). The presence of a complex of Cp with lactoferrin in breast milk was also reported (Sokolov et al., 2006). Adult and young camel Cp were isolated and purified using the Single-step chromatography on amino ethyl-activated Sepharose (Essamadi et al., 2002). However, most common purification steps of Cp include ammonium sulfate precipitation, and a series of tedious chromatographic steps such as anion-exchange chromatography, size-exclusion chromatography, and hydrophobic chromatography. Here, we report a rapid and one-step purification procedure of Cp from human plasma by AS affinity chromatography.

MATERIALS AND METHODS

Materials

Fresh human plasma was obtained from Seoul National University Hospital Blood Bank. AS was purified from the giant African snail *Achatina fulica* as previously described (Kim et al., 1996). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/diaminodipropylamine immobilization kit was purchased from Pierce (Rockford, IL, USA). Heparin, MES (2-(*N*-morpholino) ethanesulfonic acid), Trizma base, *o*-dianisidine dihydrochloride, anti-human Cp antibody, polyclonal anti-goat IgG, *o*-phenylenediamine dihydrochloride, soybean trypsin inhibitor, benzamidine were from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

Immobilization of acharan sulfate on agarose matrix and affinity chromatography procedures

AS affinity column was prepared using EDC/diaminodipropylamine immobilization kit (Pierce) according to the manufacturer's instructions. Briefly, the diaminodipropylamine-agarose matrix was washed with water followed by conjugation buffer, 0.1 M MES, 0.9% NaCl, pH 4.7. AS (250 mg) was dissolved in 25 mL of the same buffer and the solution was mixed with the gel containing the EDC solution in the shaker for 3 hr at room temperature. The column (33 cm × 2.7 cm) was packed and washed with 1 M NaCl to elute unbound AS. The amount of unbound AS was calculated by carbazole assay (Bitter and Muir, 1962). Then, the column was washed thoroughly with 20 mM Tris-HCl buffer (pH 7.4) before loading the plasma sample. All affinity chromatography procedures were performed at 4°C. Frozen human plasma (250 mL) was thawed in a 37°C water bath and then kept below 10°C. Soybean trypsin inhibitor (20 mg/L) and ben-

zamidine (5 mM) in 20 mM Tris-HCl buffer were added to human plasma to inhibit proteolysis. Upon loading the sample, the column was washed with 20 mM Tris-HCl buffer followed by the buffer containing 0.3 M NaCl until the absorbance at 280 nm was negligible. An additional stepwise elution was done with 20 mM Tris-HCl buffer containing 0.4 M, 0.7 M, and 1.0 M NaCl.

SDS-PAGE and isoelectric focusing

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970) using 10% acrylamide/Tris/HCl running gel and 4% stacking gel. The isoelectric focusing was performed with a Bio-Rad Model 111 Mini Isoelectric Focusing Cell (Hercules, CA, USA) according to the manufacturer's procedure. The protein bands were stained with a Coomassie Brilliant Blue G-250 or a silver stain kit from Bio-Rad (Hercules, CA, USA).

Western blotting

Cp resolved by SDS-PAGE were transferred to PVDF membrane in 100 mM CAPS, 10% methanol using a constant current of 30 V for overnight. The blot was incubated in a Tris-saline buffer containing 5% (w/v) non-fat milk and 0.1% (w/v) Tween 20 for 1 hr. The blot was further incubated for 2 hr with 10 mL of Tris-saline buffer containing 10 µL of goat anti-Cp antibody diluted 1 : 1000 followed by incubation for 1 hr using an anti-goat IgG. The blot was washed several times in TBS-T buffer, and it was detected by the colorimetric method using *o*-phenylenediamine as a substrate.

MALDI-TOF-MS peptide mass fingerprinting

For peptide mass fingerprinting, in-gel digestion was performed as previously described (Shevchenko et al., 1996). MALDI-TOF-MS analysis, peptides were eluted directly onto a MALDI probe with 2-cyano-4-hydroxycinnamic acid matrix solution (10 mg/mL matrix in 0.5% TFA/50% acetonitrile 1 : 1, v/v). The positive ion mode of mass spectra was acquired at a reflectron mode by a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). External calibration was performed using a standard peptide mixture of des-Arg bradykinin, angiotensin I, Glu-fibrinopeptide B, adrenocorticotrophic hormone (ACTH) clip 1-17, ACTH clip 18-39, and ACTH clip 7-38. Internal calibration was also performed using two autolysis peaks of trypsin ($[M + H]^+ = 842.50$ and 2211.10). The resulting tryptic peptides were dissolved in 0.5% trifluoroacetic acid solution, and then were desalted using ZipTipC₁₈ (Millipore, Bedford, MA, USA). Experimental masses of tryptic peptides were submitted to the search engine,

MASCOT peptide mass fingerprint software (www.matrixscience.com, London), to predict proteins based on theoretical peptide mass fragments ions. Protein score greater than 64 are significant ($p < 0.05$). The search parameters were set to consider the following modifications: *N*-terminal glutamine to pyroglutamic acid, oxidation of methionine, acetylation of protein *N*-terminus, and carbamidomethylation of cysteine.

Oxidase Activity

Cp oxidase activity was measured essentially as described by Schosinsky et al. (Schosinsky et al., 1974) using *o*-dianisidine dihydrochloride (4,4'-diamino-3,3'-dimethoxybiphenyl) as a substrate. This reagent is converted into a yellowish-brown reaction product by Cp and oxygen at pH 5. Acidification stops the enzymatic reaction, and a stable purplish-red solution that absorbs maximally at 540 nm was formed. The optimum pH of Cp was determined by varying concentration and pH of buffers.

RESULTS AND DISCUSSION

The amine-functionalized agarose matrix was linked to the carboxylic acid groups of AS for the preparation of the affinity column. The carboxyl group of AS was activated by EDC followed by the coupling of amine-functionalized agarose matrix. To determine the amount of unbound AS, the carbazole assay was performed on washing solution indicating 98.8% of AS binding to the column. Without AS immobilization on agarose matrix, all plasma proteins were eluted below 0.3 M NaCl salt concentration. After washing the column with 0.3 M NaCl, the step-wise elution was performed (Fig. 2A). Next, each fraction was analyzed on 10% SDS-PAGE, and we observed one major band and one minor band from 0.4 M NaCl fraction having molecular weights of 132 and 125 kDa, respectively. These bands were found pure in 10% SDS-PAGE (Fig. 2B, lane 5) and isoelectric focusing (pI 4.6) (Fig. 2C). The observed pI value of Cp was similar

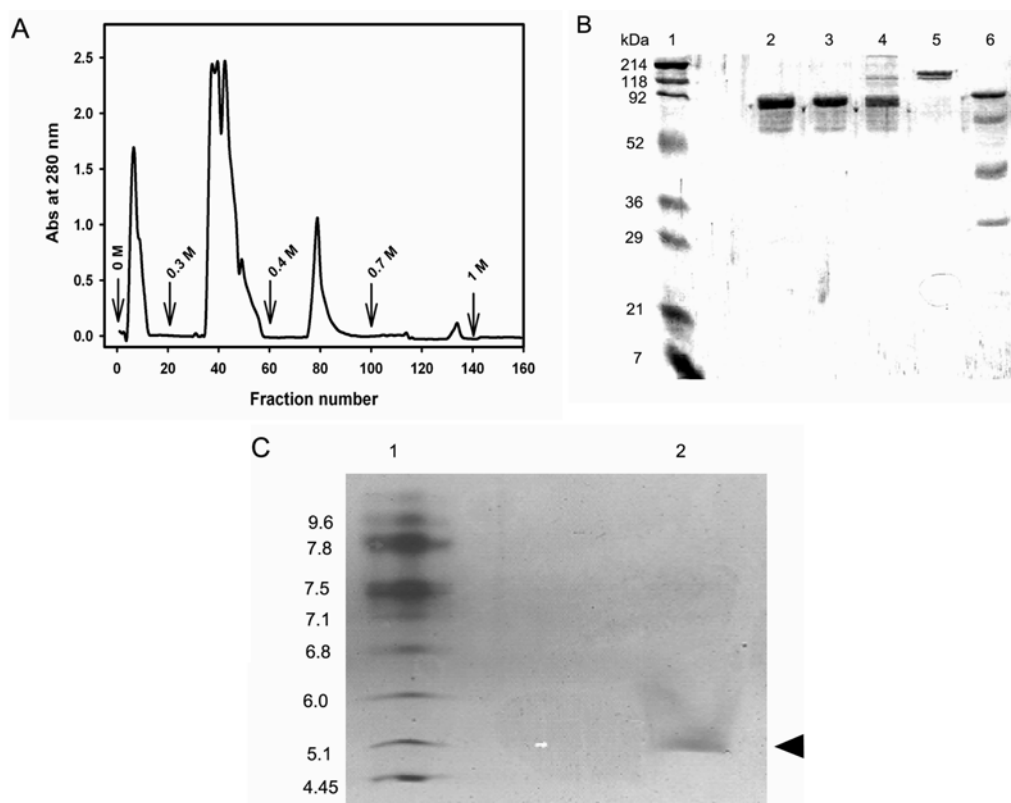


Fig. 2. **A** The elution profile of AS affinity chromatography. Cp was eluted in 0.4 M NaCl in 20 mM Tris-HCl buffer. The arrows indicate the concentration of applied salts to elute the bound proteins from column matrix. The volume of each fraction was approximately 30 mL (fractions 1-20) and 14 mL (fractions 20-160). **B** 10% SDS-PAGE of affinity chromatography fractions with Coomassie blue staining. Lane 1 is a molecular weight marker. Lane 2 is plasma sample. Lanes 3-6 are pooled proteins from the column by a stepwise salts gradient, 0 M, 0.3 M, 0.4 M, and 0.7 M, respectively. **C** Isoelectric focusing of Cp. Lane 1 is pI marker proteins (cytochrome C 9.6, lentil lectin 7.8, human hemoglobin C 7.5, human hemoglobin A 7.1, equine myoglobin minor band 6.8, human carbonic anhydrase 6.0, β -lactoglobulin B 5.1, phycocyanin 4.45). Lane 2 is Cp 100 μ g (arrowhead).

Table I. Peptide mass fingerprints by MALDI-TOF-MS on a major band (132 kDa)

No.	Access no.	Molecular weight (Da)	Mascot score ^a	Description
1	gi 1620909	115398	120	ceruloplasmin (Homo sapiens)
2	gi 4557485	122128	116	ceruloplasmin (ferroxidase)
3	gi 1070458	122574	115	ceruloplamsin ferroxidase precursor
4	gi 1942284	120009	109	X-Ray crystal structure of human ceruloplasmin
5	gi 180249	97637	77	ceruloplasmin (Homo sapiens)

^aProtein scores greater than 64 are significant ($p < 0.05$).

with the result previously reported by Narita et al. (Narita et al., 2001).

For peptide mass fingerprinting of a major band (132 kDa), the database produced positive hits (Table I), and MASCOT score greater than 64 (120, 116, 115, 109, and 77) predicted with a consistency to human Cp, Cp precursors, and Cp fragments. The highest score was 120 corresponding to a human Cp fragment of 115,398 Da. Peptide mass fingerprinting information of both bands (132 and 125 kDa) clearly allowed us to propose the identity of our purified protein as Cp. In addition, both 132 and 125 kDa bands were also detected by Western blotting using anti-Cp antiserum (Fig. 3).

Cp from man, camel, pig, rabbit, horse, and rat serum apparently have a very similar molecular weight of ~130 kDa (Ryden, 1972), which is consistent with our observation of molecular weight 132 kDa. The

propensity of human Cp to proteolytic degradation (112-115, 64-70, 48-50, and 16-20 kDa) was already reported and these fragments are relatively consistent on SDS-PAGE (Moshkov et al., 1979). Therefore, it is unlikely the minor band (125 kDa) on SDS-PAGE was a fragment. Instead, we assume the minor band more likely originated from the *N*-glycan heterogeneity. Human Cp possesses six putative *N*-glycan sites (Asn 138, 358, 397, 588, 762, and 926: from SwissProt database). The mapping of *N*-glycosylation sites of Cp by LC-MS confirmed Asn 138, 397, and 762 from human serum (Bunkenborg et al., 2004), however, no studies have been reported so far on its *N*-glycan structures or heterogeneity. Further studies would be required to establish this proposal with certainty.

The oxidase activity of purified Cp was optimum in 100 mM acetate buffer at pH 5.0 (Fig. 4). The purified Cp exhibited the oxidase activity of 200 U/L when calculated by the method of Lehmann et al. (Lehmann et al., 1974). As summarized in Table II, Cp purification generally yielded upwards of 6 mg of protein from 250 mL of human plasma. Cp was purified 106 fold with a specific activity of 0.53 U/mg protein.

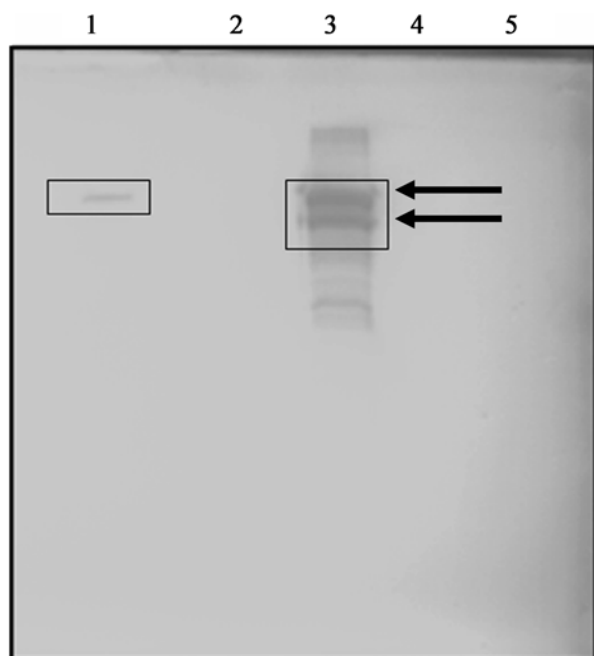


Fig. 3. Western blotting of Cp from human plasma. Lane 1 is plasma sample. Lanes 2, 3, 4 and 5 represent 0.3 M, 0.4 M, 0.7 M, and 1.0 M NaCl-eluted fractions, respectively. The arrows indicate 132 and 125 kDa Cp.

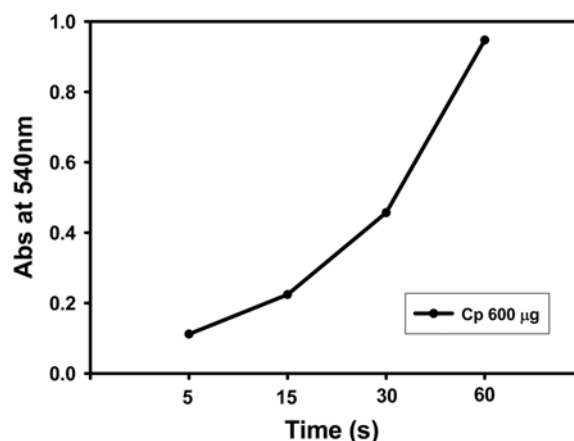


Fig. 4. Oxidase activity of purified Cp. Six hundred µg of Cp was used to measure the activity. The detailed procedure was described in the literature (Schosinsky et al., 1974) using *o*-dianisidine dihydrochloride (4,4'-diamino-3,3'-dimethoxybiphenyl) as a substrate.

Table II. Purification of Cp from human plasma

	Protein (mg)	Specific activity (U/mg)	× fold purification ^a
Plasma	17250	0.005	
0.4 M NaCl fraction	15.0	0.33	67
Desalted protein ^b	6.0	0.53	106

^aThe × fold purification was determined relative to the specific activity measured for plasma.

^bThe desalting process was done by Centricon YM-100 (MWCO 100,000 Da).

It was reported that human Cp treatment had beneficial effects on aplastic anemia patients by intravenous injection (Shimizu, 1979). However, the isolation and purification procedures of Cp are too lengthy and time-consuming, causing undesirable proteolytic degradation products. The role of AS in snails was proposed to its binding, uptake, and transport of divalent cations, especially copper and calcium (Kim et al., 1996). The blood of snails is blue, as hemocyanin is the copper-based carrier of oxygen in these animals. Our previous study showed that AS binds copper more tightly than heparan sulfate, which has a similar level of sulfation (Kim et al., 1996) that has led to the isolation of Cp by AS affinity chromatography.

In conclusion, a novel aspect of the present work is the isolation of pure Cp from human plasma in a rapid and simple fashion by taking advantage of AS-Cp interaction. This method might be a useful tool on research areas of Cp related diseases such as Wilson's disease or Menkes' disease characterized by a reduced concentration of serum Cp.

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