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Two-Stage Method for Purification of Ceruloplasmin Based on Its Interaction with Neomycin

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Abstract—A two-stage chromatography that yields highly purified ceruloplasmin (CP) from human plasma and from rat and rabbit serum is described. The isolation procedure is based on the interaction of CP with neomycin, and it provides a high yield of CP. Constants of inhibition by gentamycin, kanamycin, and neomycin of oxidase activity of CP in its reaction with *p*-phenylenediamine were assayed. The lowest K_i for neomycin (11 μM) corresponded to the highest specific adsorption of CP on neomycin-agarose (10 mg CP/ml of resin). Isolation of CP from 1.4 liters of human plasma using ion-exchange chromatography on UNO-Sphere Q and affinity chromatography on neomycin-agarose yields 348 mg of CP with 412-fold purification degree. Human CP preparation obtained with $A_{610}/A_{280} \sim 0.052$ contained neither immunoreactive prothrombin nor active thrombin. Upon storage at 37°C under sterile conditions, the preparation remained stable for two months. Efficient preparation of highly purified CP from rat and rabbit sera treated according to a similar protocol suggests the suitability of our method for isolation of CP from plasma and serum of other animals. The yield of CP in three separate purifications was no less than 78%.

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Key words: ceruloplasmin, neomycin, affinity chromatography

Ceruloplasmin (CP, ferro:O_2-oxidoreductase) is a copper-containing protein of blood plasma with $M \sim$ 132 kDa. As a positive reactant of the acute phase of inflammation [1], CP manifests a wide variety of activities as an antioxidant. Among these are the activities of ferroxidase [2], cuproxidase [3], superoxide dismutase [4], NO-oxidase [5], and glutathione peroxidase [6]. Only intact CP manifests glutathione peroxidase activity [6], facilitates iron incorporation into ferritin along a radical-free pathway [7], and inhibits the prooxidant activity of both myeloperoxidase [8] and 5-lipoxygenase [9]. In view of these facts, elaboration of an efficient purification of non-proteolyzed CP is a matter of significant interest. Preparing non-proteolyzed CP is complicated by a number of difficulties: i) the protein demonstrates extreme susceptibility to proteolytic degradation [10]; ii) one of the prevailing admixtures is prothrombin (FII) [11], the precursor of thrombin that causes the degradation of CP during storage [12]; iii) when the pH of the medium changes (goes below 5 or above 9) or when chelators are added (EDTA, 1,10-phenantroline), CP can lose its copper ions [13, 14]. The latter requires the use of buffers with neutral pH and the absence of plasma-stabilizing chelators at high concentrations. It also makes it necessary to study the properties of the sorbents used for CP purification to make sure they are unable to retain the copper of the protein.

One of the approaches for reducing of the proteolysis of CP is adding inhibitors of serine and metal-dependent proteinases to the initial plasma [15, 16]. However, phenylmethylsulfonyl fluoride (PMSF), the accepted inhibitor of serine proteinases, is quite rapidly hydrolyzed in aqueous solutions, after which it loses the capacity to inhibit serine proteinases that originate from activated proenzymes [17], whereas adding an ethanolic solution of PMSF at every step of CP purification can provoke denaturation. Metal-dependent proteinase inhibitors, such as EDTA and 1,10-phenantroline, chelate copper ions of CP, by which they decrease its activity [14]. Therefore, they can be used only in the first step of CP purification, i.e. can be added to the source plasma.

We have shown that the "spontaneous" proteolysis of CP occurring on storage produces the same spectrum of

Abbreviations: CP, ceruloplasmin; FII, prothrombin; *p*-PD, *p*-phenylenediamine.

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fragments that results from its limited proteolysis with thrombin. Besides, adding hirudin, an inhibitor of thrombin, to blood plasma before storage under sterile conditions prevents proteolysis of CP as judged by Western blotting. Thus thrombin is considered as the principal proteinase causing degradation of CP [12]. So, elimination of traces of thrombin and FII is a prerequisite condition for obtaining a CP preparation that is stable during storage.

Among the methods of isolation of CP proposed so far, the following approaches should be considered: precipitation of the protein with cooled ethanol–chloroform (9 : 1) mixture [18] and chromatography on hydroxyapatite [19], aminoethyl agarose [20], *p*-phenylenediamine (*p*-PD)-Sepharose [21], protamine-Sepharose [22], and the recently described sorbent derived from acharan sulfate [23]. The precipitation of CP by adding 2-3 volumes of cooled ethanol–chloroform (9 : 1), along with irreversible denaturation of the bulk of protein impurities, also causes some damage to CP. The more impurity-free is CP, the larger its losses upon precipitation with subsequent solubilization in buffer. This method is also inconvenient when large volumes of stock solution are employed as it requires centrifugation and selecting materials insensitive to chloroform. Besides, chloroform derivatives (e.g. phosgene) spontaneously accumulate during storage, causing cross-linking in proteins. Several research groups who purified CP by this method independently described polymeric forms of the protein [22, 24].

Chromatography on hydroxyapatite, though allowing efficient separation of isoforms of CP that differ in carbohydrate content, results in loss by the protein of some of its copper, producing the apo-form of CP [19]. Obtaining aminoethyl agarose described in several studies [20, 25] and allowing the one-stage purification of CP is reproduced with noticeable difficulties due to the hazardous conditions of synthesis at 70°C in highly alkaline medium with addition of 2-chloroethylamine generating in that milieu the volatile toxic substance ethyleneimine. The so-called "clotting effect" provided by that sorbent is its serious disadvantage manifested as coagulation of the blood plasma during chromatography [20, 26]. This effect is probably connected with retaining of FII by the resin, elimination of which is achieved by stepwise elution with calcium chloride [27]. Besides, CP eluted from the sorbent has partly reduced type I copper ions, which complicates the assessment of the main index of CP purity expressed as the relation of the absorption of copper ions to the absorption of aromatic amino acids (A_{610}/A_{280}). Only after oxidizing an aliquot of thus obtained CP with hydrogen peroxide can the index of its purity be assessed [28].

To prepare the chromatographic sorbent based on *p*-PD, a multi-stage synthesis is required. Partial oxidation of *p*-PD occurs both during the synthesis and during CP purification, which prevents the visual estimate of its retention by the resin. Besides, the latter does not provide highly purified CP, hence, to eliminate protein admixtures subsequent immunoaffinity chromatography is required [21]. Although protamine-Sepharose described in our previous paper [22] can be used to prepare pure CP, the sorbent gradually loses affinity toward CP after numerous chromatographic procedures, which is probably due to the proteolysis of protamine by plasma proteinases. In addition, the method requires that the source plasma or primarily purified CP is passed sequentially through columns with arginine- and heparin-Sepharose to eliminate FII and thrombin [22]. The recently described sorbent prepared on the basis of acharan sulfate, a polysaccharide from African snails, requires isolation of the ligand from these invertebrates. This method apparently does not provide high yield of protein as the authors described isolation of 6 mg of CP from 250 ml of plasma [23], which is not more than 10% of the CP expected in that volume. Regrettably, they neither indicated the A_{610}/A_{280} specifying the purity of CP nor did they present data on the stability of the protein, which makes it difficult to evaluate the suitability of their method.

Thus, to elaborate an alternative method of CP purification we had to reject its precipitation by organic solvents, to use cautiously metal chelators during purification, and to create a sorbent that would provide CP free of FII impurity, but would not be degraded by blood plasma components. Affinity of CP towards positively charged resins including aminoethyl agarose and the classical anion exchangers with diethylaminoethyl- and quaternary aminoethyl-groups immobilized on such sorbents as spermidine and spermine was studied [25]. The advantages of the "tentacle" structure of aminoethyl agarose obtained by matrix polymerization of ethylene imine are discussed in that work. The authors concluded that a positively charged "tentacle" sorbent is more efficient due to the tighter contact of the positively charged polymer with CP as compared to the classical ion exchangers and small cationic ligands immobilized on resins. This presumption is confirmed to a certain extent by the evidence that the positively charged polymers acharan and protamine selectively bind CP. In our search for alternative ligands applicable for CP isolation, we noted aminoglycoside antibiotics such as gentamycin, kanamycin, and neomycin. On one hand, several amino groups in their structure, invariance of the latter before and after the renal clearance, and relatively low binding of plasma proteins as judged by pharmacokinetics [29] predicted electrostatic binding of anionic CP. On the other hand, these features suggested that small amounts of other plasma proteins would be bound. Besides, the stability of sorbents including aminoglycosides against the components of the blood plasma was presumed.

Here we describe a two-stage procedure for isolation of CP from blood plasma or serum on UNO-Sphere Q and neomycin-agarose that provides high yield of purified, FII- and thrombin-free CP that is stable on storage.

MATERIALS AND METHODS

The following reagents were used in the study: skimmed dry milk, UNO-Sphere Q, Bio-Gel A-1.5m fine, horseradish peroxidase-conjugated goat antibodies against rabbit IgG (Bio-Rad, USA); triethylamine, EDTA (Merck, Germany); complete and incomplete Freund's adjuvant, sodium azide, arginine, bathocuproine, gentamycin, kanamycin, glycerol, Coomassie R-250, mercaptoethanol, ammonium persulfate, Tris (Serva, Germany); $CuSO_4 \cdot 5H_2O$, thrombin from human serum, 6-aminocaproic acid, glycine, neomycin trisulfate, *o*-dianisidine, SDS, salmon protamine, triethanolamine (TEA), *p*-PD, PMSF, 4-chloro-1-naphtol (Sigma, USA); acrylamide, N,N′-methylene-bis-acrylamide, N,N,N′N′-tetramethylethylene diamine (Laboratory MEDIGEN, Russia); Z-Ala-Ala-Arg-*p*NA·HBr (Olvex-Diagnosticum, Russia); lyophilized CP pharmaceutical (Microgen, Russia). Cyanogen bromide was obtained by bromination of KCN in a biphasic water–dichloroethane system. The solution of BrCN in dichloroethane was used for activation of the agarose gel Bio-Gel A-1.5m fine [22]. Neomycin, kanamycin, and gentamycin were immobilized by adding the antibiotics to the BrCN-activated agarose in PBS (100 mg/ml wet gel). PBS (phosphate buffer saline) was prepared as 0.15 M NaCl, pH 7.4, 1.9 mM Na_2HPO_4/8.1 mM NaH_2PO_4. Chromatographic solutions were prepared using apyrogenic deionized water with specific resistance 18.2 MΩ·cm.

Specific sorption of the purified CP on the resulting resins was assayed by the amount of protein eluted from 1 ml of wet gel. For this purpose, columns containing 1 ml of neomycin-, kanamycin-, or gentamycin-agarose equilibrated with PBS 40 ml of CP solution (0.5 mg/ml) were loaded in PBS/2. Non-bound protein was washed away by PBS/2, after which the amount of CP in eluate containing 0.5 M NaCl was determined spectrophotometrically. The coefficient $a_{610} = 0.0741$ ml/mg per cm was used [22].

Human plasma with sodium citrate (final concentration 13 mM) was used, collected after plasmapheresis of patients with gout in the toxicological ward of the Military Medical Academy (St. Petersburg). The patients gave informed consent, and the blood plasma was stored frozen. During the first working day all manipulations were carried out in a cold room (at 4-8°C). The plasma was thawed, and if fibrin precipitate spontaneous formed they were removed by filtration on a nylon filter. To 1.4 liters of filtered plasma, 0.5 μM EDTA and 2 mM 6-aminocaproic acid was added, after which the plasma was loaded on a PBS-equilibrated column packed with UNO-Sphere Q (30 × 2.5 cm). The flow rate was 10 ml/min. The column was sequentially washed free of undesired proteins with PBS and 40 mM TEA-HCl (pH 7.4) to achieve A_{280} <0.005 of the eluate. The washed column remained in a cold room overnight. The next day the column was connected to a Bio-Rad DuoFlow 20 chromatograph. Chromatography was performed using ice-cold solutions. Elution was performed at rate 2 ml/min with a linear gradient 0→0.5 M NaCl containing 40 mM TEA-HCl (pH 7.4), the total volume being 200 ml. Blue-colored fractions were pooled, diluted 10-fold with 20 mM TEA-HCl (pH 7.4), and loaded at flow rate 5 ml/min on the neomycin-agarose column (12 × 2.5 cm) equilibrated with 40 mM TEA-HCl (pH 7.4). The column was washed with 40 mM TEA-HCl (pH 7.4) to achieve $A_{280} < 0.005$ of the eluate. Proteins were eluted from the column by 120 ml of linear gradient 0→100 mM $CaCl_2$ containing 40 mM TEA-HCl (pH 7.4) at flow rate 2 ml/min. Blue-colored fractions were pooled and concentrated in a VivaSpin 20 unit. The protein concentrated 2-fold to the volume of 2 ml was diluted with 18 ml of 40 mM TEA-HCl (pH 7.4), after which its concentration was repeated. To purify CP from rabbit or rat serum, a similar protocol was used. Rats were anaesthetized with ether, and blood was collected after decapitation. The blood of rabbits was sampled from the aural vein. Serum was separated by centrifugation upon blood clotting. When the serum volume was below 0.5 liter, one working day was needed to purify CP.

At every stage of purification, fractions were tested for oxidase activity of CP with *p*-PD [30]. Total protein content was assayed by the Bradford method [31] with CP used as the standard. Homogeneity of a preparation was monitored by SDS-PAGE [32]. This was followed by Western-blotting [33] to reveal immunoreactive FII and thrombin, and activity of thrombin was assayed as well [34]. Polyclonal monovalent antibodies against FII were obtained by immunizing rabbits with CP-containing bands excised from polyacrylamide gel after SDS-free electrophoresis [22]. Oxidase activity of CP was revealed in the protein bands by staining the polyacrylamide gel with *o*-dianisidine after SDS-free electrophoresis [35, 36]. Stability of CP during storage was studied when the protein was incubated at 37°C under sterile conditions. To samples taken at various time intervals, mercaptoethanol-containing buffer was added, and the samples were boiled on a water bath before being subjected to SDS-free electrophoresis. The samples were stored at −70°C before analysis.

Thrombin activity was judged by the rate of hydrolysis of the chromogenic substrate Z-Ala-Ala-Arg-*p*NA·HBr [34]. The reaction mixture contained the substrate (0.1 mg/ml) and PBS. The value of A_{400}/min was measured upon adding an aliquot of the sample. The rate of the enzymatic reaction was expressed as NIH/ml with reference to a calibration curve obtained for thrombin with known specific activity (500 NIH/mg). NIH is the international unit of activity corresponding to the amount of thrombin causing the coagulation of 1 ml of 0.1% fibrinogen solution in 15 sec at 29°C in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.36, containing 0.66% PEG-6000.

The activity of CP in reaction with *p*-PD was determined upon incubation of a CP-containing aliquot (1-

10 μM, 1 : 100 v/v) in 0.4 M sodium-acetate buffer, pH 5.5, containing 0.05% *p*-PD. The specific activity was expressed in U per mg of total protein, where U is the estimated amount of CP in milligrams obtained by measuring A_{530} after 1 h of incubation of the samples at 37°C [30] multiplied by the empirical coefficient 0.875 mg CP/ml and by the fraction volume in milliliters.

The effect of neomycin, gentamycin, and kanamycin (12.5-50 μM) on the activity of 1 μM CP in reaction with *p*-PD (0.25-1.5 mM) was assayed by changes in the rate of the oxidized substrate formation (ΔA_{530}/min) in 0.1 M sodium-acetate buffer, pH 5.5. The values obtained with and without the presence of antibiotics were plotted in Lineweaver–Burk coordinates (1/S, 1/*v*), after which K_m, V_{max}, and K_i were calculated.

Copper content in CP preparations was determined by spectrophotometry [37]. Upon precipitation of the protein (about 5-20 μM) in solution with an equal volume of 2 M HCl and 0.6 M trichloroacetic acid followed by 5 min of centrifugation at 15,000*g*, half of the supernatant in an equal volume of 80 mM hydroxylamine and 0.44 mM bathocuproine in 2 M sodium acetate was added. Then A_{480} was measured, and the copper content was calculated using a calibration curve plotted for solutions of $CuSO_4$ (20-200 μM). Absorption spectra and enzymatic reaction rates were recorded using an SF-2000-2 OKB Spectr spectrophotometer (St. Petersburg).

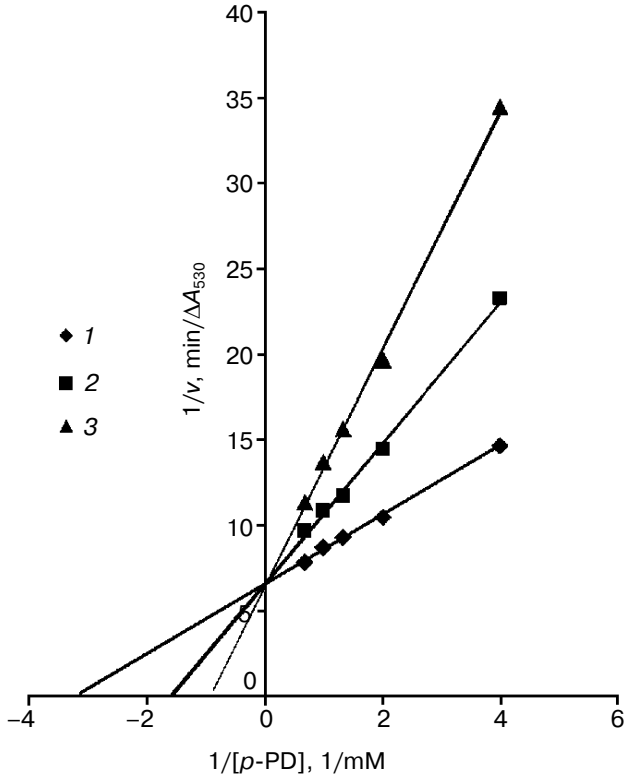

Fig. 1. Lineweaver–Burk plot of inhibiting activity of 2 μM CP in reaction with *p*-PD (0.25-1.5 mM) in 0.1 M sodium-acetate buffer, pH 5.5, in the absence (*1*) and presence of 12.5 (*2*) and 25 μM (*3*) neomycin.

RESULTS AND DISCUSSION

Experiments aimed at evaluation of the affinity of CP towards aminoglycoside antibiotics (gentamycin, kanamycin, and neomycin) revealed the strongest specific absorption of CP by neomycin-agarose (Table 1). Study of the effect of the antibiotics on the oxidase activity of CP showed that they are competitive inhibitors of its activity in reaction with *p*-PD, and they probably compete with the substrate for a common binding site (Fig. 1) as they do not alter V_{max}, but increase K_m (Table 1). It is known that the binding site for *p*-PD is separate from that for Fe(II) and biogenic amines. The latter is located in domain 6 of CP, while the former is found in domain 4 and includes amino acid residues M668 and W669 close to the type I copper ion [38]. Aminoglycosides within the series gentamycin, kanamycin, neomycin feature respectively increasing number of primary amino groups, i.e. 2,

Table 1. Parameters of interaction of CP with aminoglycoside antibiotics

Parameter	Without antibiotics	Gentamycin	Kanamycin	Neomycin
a, mg/ml sorbent	–	2.7 ± 0.3	5.6 ± 0.4	10 ± 0.5
K_i, μM	–	132.8 ± 1.4	43.9 ± 0.3	11.2 ± 0.8
K_m, μM	311 ± 12	369 ± 20 (25) 429 ± 14 (50)	400 ± 13 (12.5) 487 ± 9 (25)	626 ± 14 (12.5) 1076 ± 25 (25)
V_{max}, A_{530}/min	0.15 ± 0.01	0.15 ± 0.01 (25) 0.15 ± 0.01 (50)	0.15 ± 0.01 (12.5) 0.15 ± 0.01 (25)	0.15 ± 0.01 (12.5) 0.15 ± 0.01 (25)

Note: *a*, CP specific adsorption; K_i, mean value obtained for effect of two concentrations of an antibiotic on CP activity in reaction with *p*-PD. K_i was calculated on the basis of K_m and V_{max}, represented in the two lines; concentrations of antibiotics in μM are given in brackets.

Table 2. Data on steps of human CP purification

Stage (fraction)	V, ml	m, mg	a_1, U/mg	n	a_2, NIH/g	A_{610}/A_{280}	η, %
1 (plasma with citrate)	1400	122 821	0.00241	–	0.46	–	100
2 (fractions from UNO-Sphere Q)	53	2401	0.0883	37	19.9	0.0065	72
3 (fractions from neomycin-agarose)	40	350	0.994	412	<0.03	0.052	118

Note: V, volume; m, mass of total protein (based on results of total protein measurements with CP used as standard [31]); a_1, CP specific activity (based on measurements of activity in reaction with *p*-PD, where U is estimated amount of CP in mg obtained by measuring A_{530} after 1 h of incubation of CP aliquot (1/100) with *p*-PD under conditions described previously [30] multiplied by empirical coefficient 0.875 ml/mg for pure CP and by fraction volume in ml); n, purification fold; a_2, thrombin specific activity (based on measurements of activity towards Z-Ala-Ala-Arg-*p*NA [34]); η, yield (based on measurements of activity in reaction with *p*-PD).

4, and 6, which corresponds to the decrease in K_i and the growth of the specific absorption of CP. Therefore, neomycin-agarose was chosen as the preferred sorbent for subsequent experiments with CP purification.

Data on the steps of purification of human CP are summarized in Table 2. The first stage yielded 72% of CP assayed in the source plasma, as judged by the oxidase activity with *p*-PD. However, at the next stage the total oxidase activity was higher than that measured in the source plasma. This probably occurred due to the underestimation of the oxidase activity resulting from the presence of sodium citrate in the plasma used. Upon loading CP on neomycin-agarose its absorption was easily monitored as the intensity of the blue band on the resin, attributed to the color of type I copper ions in CP, increased. Elution of CP was seen as a dark-blue band of the eluted protein moving down the column (Fig. 2a). The specific dynamic absorption of human CP was close to 10 mg CP per ml of neomycin-agarose. Since the activity of CP did not decrease during purification, it can be concluded that the chromatography did not result in loss of the copper ions needed for CP to perform as an oxidase. Indeed, measuring copper content in the preparation gave the value 5.9 ± 0.1 mole Cu per mole CP, which is in agreement with the published data. A protein band with M 132 kDa corresponding to the non-degraded CP was predominant in the preparation as judged by SDS-PAGE (Fig. 2b).

Assay for FII in fractions showed that traces were present in the preparation obtained by chromatography on UNO-Sphere Q. In probing experiments with NaCl linear gradient elution of proteins absorbed on neomycin-agarose, partial overlap of the first-eluted FII peak and the following peak of CP was observed. However, upon elution with gradient concentrations of $CaCl_2$ the FII impurity was released from the resin much earlier. Western-blotting with antibodies against FII showed that neither FII nor thrombin was present in the CP-containing fractions (Fig. 2c). Active thrombin was found only in the source plasma and in fractions eluted from UNO-Sphere Q, but not in the CP preparation purified on neomycin-agarose. The purity index A_{610}/A_{280} for CP thus obtained was 0.052. Therefore, the protein we obtained had the grade of purity no less than preparations isolated previously by other methods [20, 26]. The procedure for CP isolation from varying volumes of plasma (0.9-1.5 liters) was repeated five times, and each preparation of CP had the same grade of purity.

Storage of the CP at 37°C under sterile conditions did not result in proteolytic degradation of the protein for at least two months (Fig. 2d). Therefore, the preparation contained neither thrombin nor other proteinases capable of degrading CP.

The data concerning CP isolation from sera of rats and rabbits according to the same protocol are summarized in Table 3. In both cases, the protein yield was above 78% and the preparations were homogeneous as judged by SDS-PAGE (Fig. 2b). The index of purity A_{610}/A_{280} for rabbit CP was 0.049 and for rat CP it was 0.054, which is better than the best values described so far, respectively, 0.0475 [39] and 0.051 [40]. The specific binding capacity of neomycin-agarose towards rat and rabbit CP was 3-5 mg of the protein per ml of the sorbent, which is lower than the respective value for human CP. Purification of CP from rat serum was repeated four times, and the rabbit protein was purified three times. In each repeat the purity indices were the same.

A pharmaceutical composed of lyophilized CP produced by FSUE SIA Microgen (Ministry of Public Health of the Russian Federation) approved in 1990 by the Pharmacological Committee (register number 90/411/1) is known today. Laboratory tests of this preparation showed its heterogeneity (Fig. 3, a and b) and low index of purity $A_{610}/A_{280} = 0.0087$. Both in blood plasma and in preparations purified according to our method non-degraded CP with M ~ 132 kDa prevailed, while its proteolytic fragments contribute not more than 10% (Fig. 3a). In contrast, the pharmaceutical CP contains 10% non-degraded protein with M ~ 132 kDa, but is composed mostly of proteolytic fragments with smaller M:

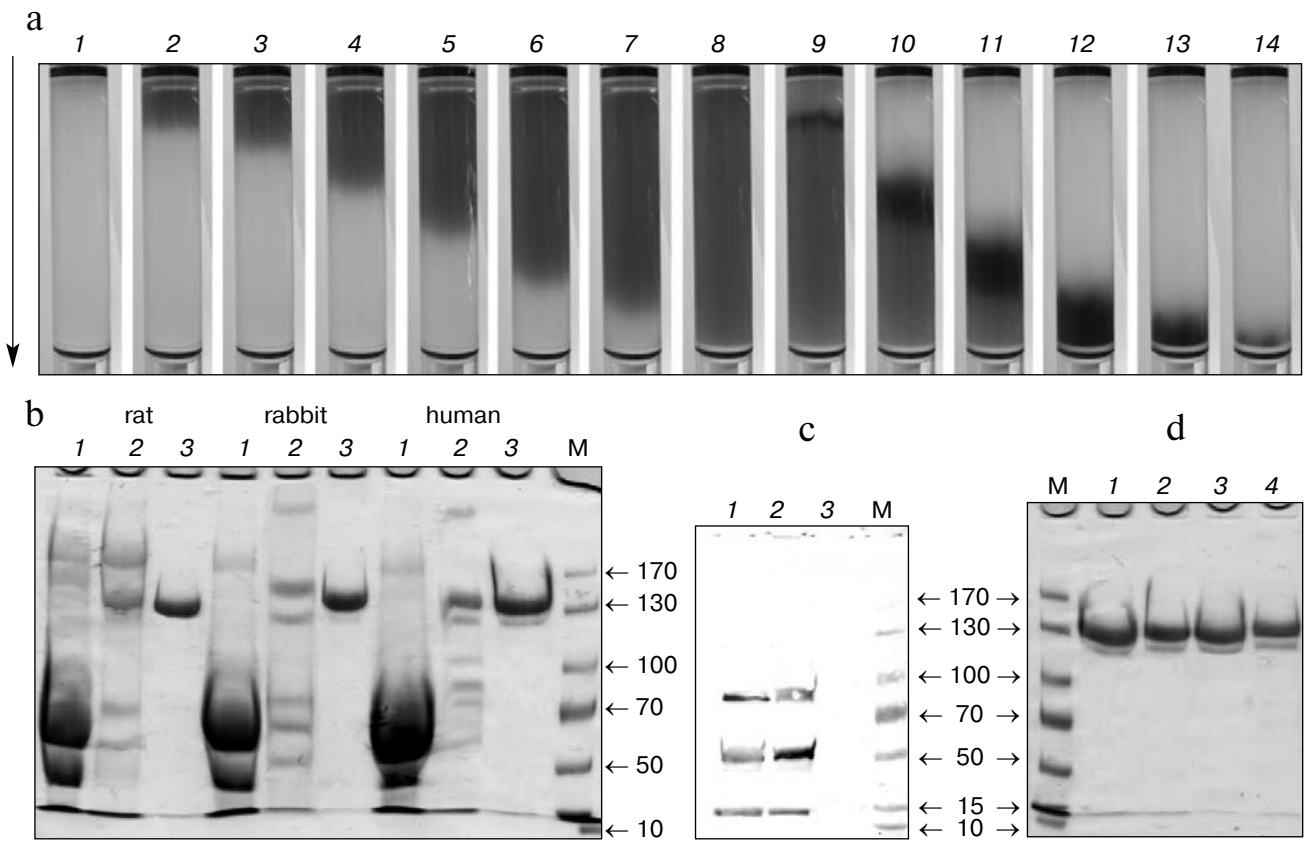

Fig. 2. Steps of purification of rat, rabbit, and human CP. a) Column with neomycin-agarose during chromatography of human CP obtained from UNO-Sphere Q: *1*) before loading of CP-containing fraction; *2-7*) progress of the blue front of the adsorbed CP; *8*) column after washing away unwanted proteins; *9-11*) progress of CP eluted with $CaCl_2$; *12-14*) yield of CP-containing fraction. Arrow shows the direction of flow. b, d) SDS-PAGE (Coomassie R-250 staining). c) Western-blotting with antibodies to FII (1 : 10,000, horseradish peroxidase-labeled secondary antibodies 1 : 5000, staining with 4-chloro-1-naphtol and H_2O_2), 50 μg of protein per lane: *1*) source human plasma; *2*) pooled CP-containing fractions eluted from UNO-Sphere Q; *3*) pooled CP-containing fractions eluted from neomycin-agarose. d) CP preparation during storage at 37°C under sterile conditions: *1*) upon purification; *2*) after 1 week; *3*) after 1 month; *4*) after two months. M, colored molecular mass markers, arrows indicating *M* in kDa.

110, 67, 52 kDa and less (Fig. 3a). Proteolyzed CP loses its important antioxidant features. Moreover, lyophilization of CP causes the loss of its copper ions. Assay of the copper content in the pharmaceutical CP showed that ca. 75% of the metal in the preparation does not account for active protein. As a result, such CP has a decreased oxidase activity, which is confirmed by *o*-dianisidine staining of the gel after disc-electrophoresis (Fig. 3c). The specific activity of CP purified according to our protocol is 0.994 U/mg, while the same figure for the pharmaceutical CP is 0.195 U/mg. Hence, the lyophilized CP demonstrates five times lower activity with the conventional substrate *p*-PD.

Because CP is a pharmaceutical and is an object of intense scientific studies (more than 7500 papers were cited in PubMed by December, 2011), elaboration of an efficient method for isolation of non-proteolyzed CP with perspectives of using the method in pharmaceutical industry is a matter of topical interest.

Our method of CP isolation is convenient as UNO-Sphere Q, neomycin, and the resins activated to attach primary amino-containing ligands are commercially available. The procedure for the sorbent synthesis and chromatography is easily reproduced in the laboratory and does not require additional fractionation by organic solvents and centrifugation. The isolation conditions allow using buffer solutions with physiological pH, which are not likely to damage CP isolated from plasma/serum of other vertebrates. Copper ions in CP of other vertebrates are usually more labile than those in human CP. However, successful purification of CP from rat and rabbit sera suggests our method for isolation of CP of other vertebrates. A significant advantage of the proposed method is that there is no need to dilute the source plasma in the first stage of CP isolation, while its isolation on diethyl aminoethyl-resins, aminoethyl-agarose and *p*-phenylenediamine-Sepharose requires that the initial material is diluted 3-20-fold, which considerably increas-

Table 3. Data on CP purification from human plasma (I) and from rat (II) and rabbit sera (III)

Parameter	I	II	III
Initial volume of serum/plasma, ml	1400	100	100
Amount of CP obtained, mg	348	54.4	31.9
Purification, fold	412	352	365
Specific activity of thrombin, NIH/g	<0.03	<0.03	<0.03
A_{610}/A_{280} prior to neomycin-agarose	0.007	0.006	0.003
A_{610}/A_{280} after neomycin-agarose	0.052	0.054	0.049
Mole copper per mole CP	5.9	6.1	6.0
Yield as judged by activity in reaction with *p*-PD, %	113	83	79

Note: Based on spectrophotometric measurements using coefficient a_{610} = 0.0741 ml/mg per cm.

es the time both for the absorption of the protein on a column and its contact with the extraneous components of plasma. The absence of the necessity to dilute plasma with our method is based on the ability of UNO-Sphere Q to absorb efficiently the target protein from undiluted plasma or serum at flow rate 10 ml/min and higher. The sorbent also allows rigorous washing, which does not result in degradation of its properties. Since CP-containing fractions eluted from the first sorbent are diluted to a volume comparable only to 1/3 of the initial material, the

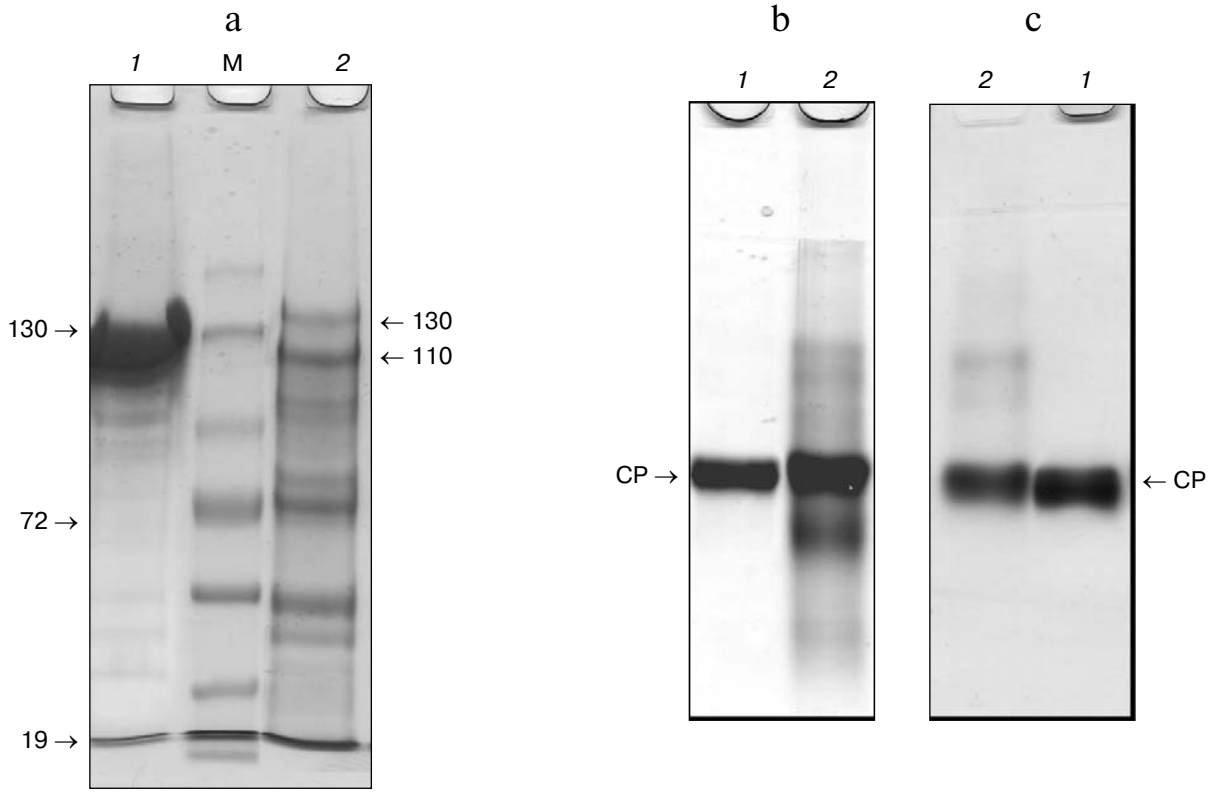

Fig. 3. Comparing human CP purified on neomycin-agarose (*1*) with pharmaceutical CP (*2*). a) SDS-PAGE (Coomassie R-250 staining); 50 μg protein per lane. M, colored molecular mass markers, arrows indicate *M* in kDa. b, c) Disc-electrophoresis in SDS-free polyacrylamide gel (Coomassie R-250 staining (b), *o*-dianisidine staining (c)); 50 μg protein per lane.

total time needed to load CP and to elute the undesired proteins from neomycin-agarose is not longer than 2 h. Using the first stage also diminishes the probability of contamination of CP with plasma components and does not require rigorous washing of the column after multiple chromatographic procedures on neomycin-agarose. Another advantage of our method is the possibility to store at 4°C the columns with CP adsorbed on UNO-Sphere Q and neomycin-agarose after washing away the undesired proteins. Such storage for at least one week does not result in decrease in the protein yield or in any noticeable decrease in its activity. The procedure for isolation of human CP described here can be used for industrial production of CP preparations.

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