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Identification of Complexes Formed by Ceruloplasmin with Matrix Metalloproteinases 2 and 12

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Abstract—Marked sensitivity to proteolytic degradation results in the loss of multiple antioxidant properties of ceruloplasmin (CP), the multicopper oxidase of mammalian plasma. In this study, gel filtration of virtually pure CP (purity 99.7%) yielded complexes of this protein. Subjecting the complexes to SDS-free PAGE revealed other proteins along with CP. These were identified as matrix metalloproteinases (MMP-2 and MMP-12) by means of tryptic fragment mass spectrometry. Electrophoretic bands corresponding to MMP-2 (72 and 67 kDa) and MMP-12 (22 kDa) displayed gelatinase activity. The identified proteinases contained heparin-binding motifs inherent in the complex-forming partners of CP, such as lactoferrin, myeloperoxidase, and serprocidines. Therefore, admixtures of MMPs can be efficiently eliminated from CP preparations by chromatography on heparin-Sepharose as proposed previously.

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Ceruloplasmin (CP; EC 1.16.3.1) is a copper-containing glycoprotein with molecular mass ~132 kDa. When attacked by proteinases, human CP is degraded with extreme ease. This peculiarity along with the presence of three homologous amino acid sequences within the protein chain of CP had been for more than 30 years since the protein was discovered in 1944 the basis of discussions whether its molecule is a single-chain structure or a set of homologous subunits [1]. Isolation of non-degraded and stable CP remains rather difficult [2]. Many authors report the presence in CP preparations of prothrombin [3] and non-identified metalloproteinase [4] and suggest that protease inhibitors be used at every step of CP purification [5].

CP is a universal antioxidant displaying several types of oxidase activity. Accomplishing four-electron transfer to oxygen with water as the end-product, CP oxidizes Fe^{2+} [6] and Cu^{+} [7], thus preventing free radical formation according to the Fenton and Haber–Weiss mechanism. CP was shown to possess activity of superoxide dismutase [8]. Non-degraded CP shows glutathione-dependent peroxidase activity [9]. It was shown that CP is a plasma NO-oxidase that converts NO into NO^{+}, the latter becoming nitrite in the presence of water. NO-oxidase activity of plasma is noticeably diminished by immunoprecipitation of CP. Hence, nitrite concentration in patients with inborn deficiency of the CP gene is two times lower [10].

Since the early 1990s, interactions of CP with other proteins have been demonstrated; this has broadened our views on its antioxidant functions. Non-degraded CP favors the oxidation of prooxidant ferrous iron and loading of Fe^{3+} in ferritin [11]. An isoform of CP fixed on astrocyte membranes interacts with ferroportin 1 and thus promotes Fe^{2+} egress from cells [12]. When CP interacts with lactoferrin, its oxidase activity increases [13]. CP forms a complex with myeloperoxidase inhibiting its chlorinating activity and thus preventing the prooxidant activity of this leukocytic enzyme [14]. According to our data, it is only the non-degraded CP that possesses a pronounced inhibiting effect [15, 16].

From these data, it becomes clear that to exercise its multiple anti-oxidant functions CP needs the integrity of its polypeptide chain. However, despite many years of study the question about the nature of proteinase degradation of CP has found no answer.

The goal of this study was to identify the CP complex components isolated by gel filtration from virtually pure

Abbreviations: CP, ceruloplasmin; MMP, matrix metalloproteinases.

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CP preparation (99.7% purity). Here we describe for the first time complexes of CP with matrix metalloproteinases, i.e. MMP-2 and MMP-12.

MATERIALS AND METHODS

The following reagents were used: cyanogen bromide (Fluka, Switzerland); triethylamine and EDTA (Merck, Germany); Sepharose 4B, DEAE-Sephadex A-50, and Sephadex G-200 superfine (Pharmacia, Sweden); trypsin (Promega, USA); sodium azide, human serum albumin, glycerol, gelatin, Coomassie R-250, mercaptoethanol, ammonium persulfate, Triton X-100, and Tris (Serva, Germany); 2,5-dihydroxybenzoic acid, glycine, SDS, molecular mass markers, salmon protamine, Folin reagent, phenylmethylsulfonyl fluoride (PMSF), and 4-chloro-1-naphthol (Sigma, USA); acrylamide, arginine, N,N′-methylene-bis-acrylamide, and N,N,N′N′-tetramethylethylenediamine (Laboratory MEDIGEN, Russia); PBS (phosphate buffered saline) (0.15 M NaCl, pH 7.4, 1.9 mM Na_2HPO_4/8.1 mM NaH_2PO_4).

Arginine and protamine (10 and 4 mg, respectively, per ml of wet gel) were immobilized on BrCN-activated Sepharose 4B [17]. Protein content was assayed in three parallel samples by the Folin–Lowry procedure [18] with human serum albumin used as the standard. Spectra were registered on SF 2000-02 (OKB Spektr, Russia).

CP preparation was isolated from blood plasma by chromatography on protamine-Sepharose as described previously [17]. Minor modifications of the method were adopted, i.e. CP isolated from 1 liter of citrate-containing blood plasma was subjected to the primary purification using ion-exchange chromatography on DEAE-Sephadex A-50. The plasma was diluted two times with 50 mM sodium acetate buffer containing 1 μM PMSF (pH 5.5), loaded on a column equilibrated with 50 mM sodium acetate buffer, after which protein was eluted with linear gradient 0→400 mM NaCl in 50 mM sodium acetate buffer (pH 5.5). Upon adding two volumes of ethanol–chloroform (9 : 1 v/v) cooled to –70°C to the blue fractions, these were centrifuged at 6000*g* (4°C). The blue precipitate was dissolved in PBS, after which CP was separated from denatured admixtures by centrifugation at 15,000*g* (4°C). The supernatant was eluted through a column with arginine-Sepharose equilibrated with PBS and then loaded on protamine-Sepharose. The column was washed with PBS, after which CP was eluted by 1 M NaCl in 10 mM sodium phosphate buffer, pH 7.4. Virtually pure CP (A_{610}/A_{280} = 0.050; 99.7% purity) was subjected to gel filtration on a column with Sephadex G-200 superfine (90 × 2.5 cm) equilibrated with PBS. Fractions (1 ml) corresponding to separate peaks were pooled and concentrated by centrifugation at 2000*g* (4°C) in a Vivaspin 20 cell with a filter retaining proteins with molecular mass above 10 kDa.

Molecular masses of proteins and proteolytic fragments of CP were determined by SDS-PAGE [19]. CP was visualized in gel after electrophoresis under nondenaturing conditions [20] by coloring with the specific chromogenic substrate *o*-dianisidine [21].

Mass spectra were measured using a Bruker (Germany) spectrograph at the Research Institute of Physico-Chemical Medicine (Moscow). To prepare samples, the proteins were resolved by PAGE and the protein-containing gel pieces were cut out. Tryptic hydrolysis of proteins in polyacrylamide gel was done as follows. A gel piece of 1 mm^3 was washed twice for 30 min in 100 μl of 40% acetonitrile solution in 0.1 M NH_4HCO_3 at 37°C to remove the dye. After washing, 100 μl of acetonitrile was added to each piece. Then the acetonitrile was eliminated, a gel piece dried, and 4 μl of solution containing modified trypsin (15 μg/ml) in 0.05 M NH_4HCO_3 was added. Hydrolysis lasted for 18 h at 37°C, after which 8 μl of 0.5% trifluoroacetic acid in 10% aqueous solution of acetonitrile was added with thorough mixing. The solution above the precipitated gel was used to obtain MALDI mass spectra. Samples for mass spectrometry were prepared as follows: 1 μl of sample solution was mixed on a probe with 0.3 μl of 2,5-dihydroxybenzoic acid (10 mg/ml in 20% aqueous solution of acetonitrile with 0.5% trifluoroacetic acid). The resulting mixture was dried in air. Mass spectra were obtained on an Ultraflex II tandem MALDI-TOF spectrometer (Bruker) equipped with a UV laser (Nd). Mass spectra were acquired in positive ion reflector mode with 0.005% accuracy of mass determination after adjusting the calibration on trypsin autolysis peaks. Peptide fingerprints obtained were analyzed online by the MASCOT program (http://www.matrixscience.com). Peptide fingerprints were screened among human proteins in the NCBI database with the precision indicated, possible methionine oxidation by atmospheric oxygen, and the likely modification of cysteines by acrylamide being accounted for.

Gelatinase activity *in situ* was revealed upon electrophoresis of samples in SDS-containing polyacrylamide gel with 0.1% gelatin [22]. Prior to electrophoresis, samples were mixed with SDS-containing application buffer. However, they were neither heated nor treated with S–S bond reducing substances. After electrophoresis, the polyacrylamide gel was washed three times for 15 min in 2.5% Triton X-100 and incubated for 18 h in 200 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.4, at 37°C. Then the gels were fixed and stained with Coomassie R-250. Gelatinase activity was judged by the absence of background coloring.

RESULTS AND DISCUSSION

Gel filtration on Sephadex G-200 of 300 mg of CP purified to 99.7% gave a peak containing 2 mg of protein (Fig. 1; fractions 1-3), which is less than 1% of CP

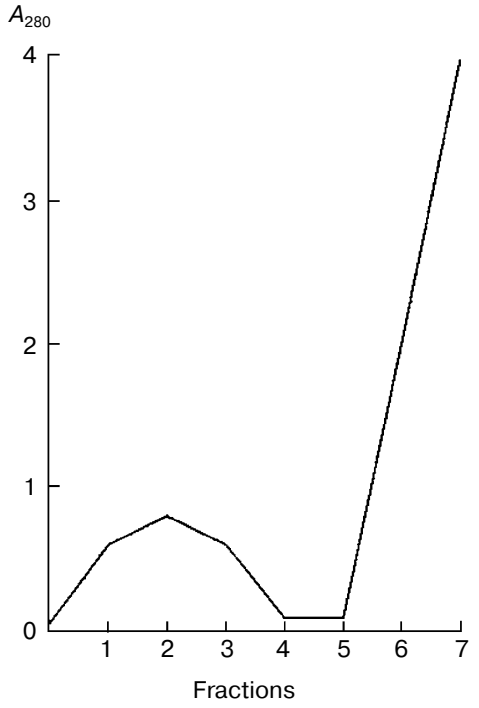

Fig. 1. Analysis of proteins eluted from Sephadex G-200 upon gel filtration of CP preparation. Fraction 5 – the beginning of the main peak of CP (contains 99.3% of the protein loaded on the column). Fraction volume, 1 ml.

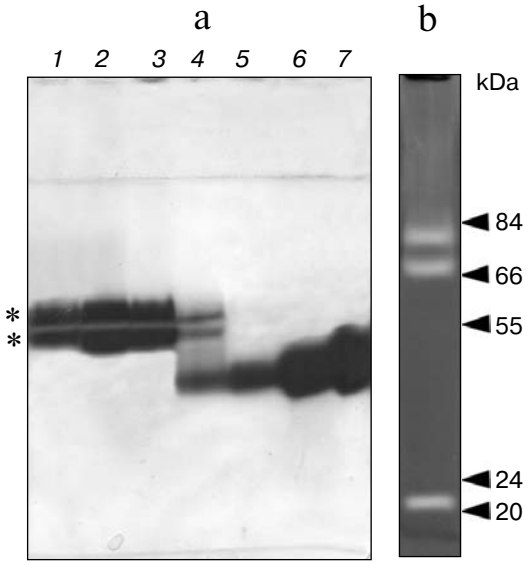

Fig. 2. PAGE of protein fractions obtained by gel filtration of GP preparation on Sephadex G-200. a) SDS-free PAGE (*o*-dianisidine staining). Lanes: *1-7*) fractions 1-7 (20 µl). Asterisks mark the bands subjected to mass spectrometry. b) Analysis of gelatinase activity *in situ* in fraction 2 (2 µl) (SDS-PAGE; Coomassie R-250 staining). Arrows show the positions of protein markers (kDa).

applied on the column, eluted ahead of the main peak (Fig. 1; the first part of the main peak is shown starting from fraction 5). In the earlier peak, SDS-free electrophoresis revealed the bands that were colored by a chromogenic substrate of CP, *o*-dianisidine, though it had electrophoretic mobility different from that of CP from the main peak (Fig. 2a). So-called dimeric isoforms of CP with molecular mass about 200 kDa were described previously [23]. If we suggest that the fast-eluting peak contains CP dimers, the spectral features of the latter should have no noticeable differences from those of monomeric CP. However, the ratio between the absorbance of Cu(I) and that of aromatic amino acids (A_{610}/A_{280}) accepted as the index of CP purity was 0.027 ± 0.001 in the fast-running fraction, which is about two times lower than such ratio in the main peak, i.e. 0.051 ± 0.001 typical of homogeneous CP. Moreover, dimeric structure gave no explanation to the occurrence of two electrophoretic CP-containing bands with similar mobility (Fig. 2a). Hence, we suggested that the latter contain two complexes of CP that have almost the same electrophoretic mobility.

Mass spectrometry of proteins in the CP-containing slow-migrating bands (Fig. 2a*) showed that along with the tryptic fragments of CP, the bands include amino acid stretches belonging to MMP-2 and MMP-12, two metalloproteinases (table).

Gelatinase activity revealed *in situ* after SDS-PAGE (see "Materials and Methods") confirmed the presence of MMPs in the preparation of CP complexes (Fig. 2b). The bands with molecular masses 72, 67, and 22 kDa behaved as a gelatinase, which is indicated by complete absence of colored gelatin in the respective sites of the gel. The bands with molecular masses 72 and 67 kDa correspond to the proenzyme and the active MMP-2, respectively, while the 22-kDa band conforms to the active MMP-12 [24].

In the resulting preparations of CP complexes, we found a 19-kDa band corresponding to the fragment split first (F5 fragment) in the course of spontaneous proteolysis of CP [25]. The band was identified as F_5 by mass spectrometry of its tryptic fragments (table). MS/MS spectra of two peptides with molecular masses 1565.77 and 2668.23 Da confirmed their identity with the amino acid sequence of F5. One should note that no high molecular mass fragments of CP spontaneous hydrolysis were found in the preparation.

It is noteworthy that no single proteinase regarded as an enzyme likely to hydrolyze CP (plasmin, elastase) would cut this protein so as to produce the set of fragments originating upon spontaneous proteolysis. As a rule, the polypeptide chain of CP would split into smaller fragments that did not have molecular masses of those appearing spontaneously.

Hence, one of the reasons for the proteolysis occurring in CP preparations is complex formation of the latter

Peptide fingerprint of CP complexes and of a CP fragment with molecular mass 19 kDa (results of MASCOT search (http://www.matrixscience.com))

Beginning–end	Expected *M* value, Da	Computed *M* value, Da	Δ, Da	Peptide
MMP-2 chain A (gelatinase A, 71 kDa, 19% of sequence found)				
8-18	1173.57	1173.60	–0.03	K.FPGDVAPKTDK.E
16-33	2088.96	2089.02	–0.06	K.TDKELAVQYLNTFYGCPK.E
73-86	1612.78	1612.71	0.07	R.CGNPDVANYNFFPR.K
133-146	1586.75	1586.75	0	R.IHDGEADIMINFGR.W
242-263	2356.04	2356.01	0.03	K.YGFCPHEALFTMGGNAEGQPCK.F
300-330	3298.53	3298.48	0.05	K.YGFCPETAMSTVGGNSEGAPCVFPFTFLGNK.Y propionamide (C)
331-343	1430.66	1430.61	0.05	K.YESCTSAGRSDGK.M propionamide (C)
MMP-12 catalytic domain (22 kDa, 23% of sequence found)				
1-6	744.38	744.37	0.01	-.MGPVWR.K
7-13	979.51	979.52	–0.01	R.KHYITYR.I
8-13	851.41	851.43	–0.02	K.HYITYR.I
14-23	1252.53	1252.55	–0.02	R.INNYTPDMNR.E oxidation (M)
48-61	1518.76	1518.82	–0.06	K.INTGMADILVVFAR.G
F5 fragment of CP (19 kDa, 27% of sequence found)				
31-38	975.46	975.43	0.03	K.TYSDHPEK.V
39-51	1565.76	1565.72	0.04	K.VNKDDEEFIESNK.M
103-125	2668.23	2668.25	–0.02	R.GVYSSDVFDIFPGTYQTLEMFPR.T
103-125	2684.23	2684.25	–0.02	R.GVYSSDVFDIFPGTYQTLEMFPR.T oxidation (M)

with MMP-2 and MMP-12. This is not the first example of CP interacting with proteinases, since we showed previously among neutrophilic proteins CP interacts with three homologous proteinases, i.e. elastase, cathepsin G, and proteinase 3 [26]. Like the majority of proteins interacting with CP (such as lactoferrin, myeloperoxidase, protamine, elastase, cathepsin G, proteinase 3, and azurocidin), MMP-2 and MMP-12 display affinity towards heparin. This feature justifies the chromatography on heparin-Sepharose that we proposed to purify a stable CP preparation [17].

The loop ^{885}TLKVFQPRRK^{894} linking CP domains 5 and 6 is the first site of proteolytic degradation. On one hand, this results from the presence of several proteinase-susceptible bonds in it. Trypsin-like proteinases (plasmin) cleave post-K and -R peptide bonds, elastase-like proteinases (elastase, proteinase 3) cut bonds with N-terminal L and V, while chymotrypsin-like proteinases (cathepsin G) split the protein chain after every F. On the other hand, this loop has high mobility and poorly ordered structure that is not "resolved" even when highly purified preparations of non-degraded CP are used for X-ray studies [27]. Splitting this loop is enough for CP to lose its glutathione-dependent peroxidase activity [9], the capacity to efficiently load Fe^{3+} into ferritin [11], and to inhibit safely the prooxidant features of myeloperoxidase [15, 16].

It can be suggested that proteolytic degradation of CP is one of the biochemical mechanisms resulting in diminishing the antioxidant state of an organism. This notion is supported by the fact that increased proteolytic activity of blood plasma in hemophilic patients results in a drop of antioxidant indices and to pronounced oxidative stress, while CP in such plasma is subjected to noticeable proteolytic degradation [28].

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