
**Free Radical Research**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713642632

# Ceruloplasmin and myeloperoxidase in complex affect the enzymatic properties of each other

Alexej V. Sokolov [a]; Kira V. Ageeva [a]; Maria O. Pulina [a]; Olga S. Cherkalina [a]; Valeria R. Samygina [b]; Irina I. Vlasova [c]; Oleg M. Panasenko [c]; Elena T. Zakharova [a]; Vadim B. Vasilyev [a]
[a] Department of Molecular Genetics of the Institute for Experimental Medicine of the Russian Academy of Medical Sciences, Saint-Petersburg, Russia [b] A. V. Shubnikov Institute of Crystallography of the Russian Academy of Sciences, Moscow, Russia [c] Research Institute of Physico-Chemical Medicine, Moscow, Russia

Online Publication Date: 01 November 2008

**To cite this Article** Sokolov, Alexej V., Ageeva, Kira V., Pulina, Maria O., Cherkalina, Olga S., Samygina, Valeria R., Vlasova, Irina I., Panasenko, Oleg M., Zakharova, Elena T. and Vasilyev, Vadim B.(2008)'Ceruloplasmin and myeloperoxidase in complex affect the enzymatic properties of each other',Free Radical Research,42:11,989 — 998

**To link to this Article: DOI:** 10.1080/10715760802566574

**URL:** http://dx.doi.org/10.1080/10715760802566574

*Free Radical Research*, November–December 2008; 42(11–12): 989–998

informa
healthcare

# Ceruloplasmin and myeloperoxidase in complex affect the enzymatic properties of each other

ALEXEJ V. SOKOLOV[1], KIRA V. AGEEVA[1], MARIA O. PULINA[1], OLGA S. CHERKALINA[1], VALERIA R. SAMYGINA[2], IRINA I. VLASOVA[3], OLEG M. PANASENKO[3], ELENA T. ZAKHAROVA[1], & VADIM B. VASILYEV[1]

[1]*Department of Molecular Genetics of the Institute for Experimental Medicine of the Russian Academy of Medical Sciences, Saint-Petersburg, Russia,* [2]*A. V. Shubnikov Institute of Crystallography of the Russian Academy of Sciences, Moscow, Russia, and* [3]*Research Institute of Physico-Chemical Medicine, Moscow, Russia*



Accepted by Dr H. Sies

*(Received 21 August 2008; revised 12 October 2008)*

**Abstract**
Ceruloplasmin (CP), the multicopper oxidase of plasma, interacts with myeloperoxidase (MPO), an enzyme of leukocytes, and inhibits its peroxidase and chlorinating activity. Studies on the enzymatic properties shows that CP behaves as a competitive inhibitor impeding the binding of aromatic substrates to the active centre of MPO. The contact between CP and MPO probably entails conformational changes close to the *p*-phenylenediamine binding site in CP, which explains the observed activation by MPO of the substrate's oxidation. CP subjected to partial proteolysis was virtually unable to inhibit activity of MPO. The possible protein–protein interface is comprised of the area near active site of MPO and the loop linking domains 5 and 6 in CP. One of the outcomes of this study is the finding of a new link between antioxidant properties of CP and its susceptibility to proteolysis.

**Keywords:** *Ceruloplasmin, myeloperoxidase, protein–protein interaction, limited proteolysis, inhibitory enzyme, activating enzyme*

**Abbreviations:** *ABTS, 2,2′-azino-di(3-ethylbenzthiazolinesulphonic acid) disodium salt; CP, ceruloplasmin; LF, lactoferrin; MPO, myeloperoxidase; p-PD, p-phenylenediamine.*

## Introduction

Ceruloplasmin (CP; ferro $O_2$-oxidoreductase; EC 1.16.3.1, $M \sim 132$ kDa) is the copper-containing oxidase of mammalian blood plasma. It is capable of oxidizing Fe (II) [1] and Cu (I) [2]. Normally the reaction cycle is composed of a four-electron transfer onto oxygen, water being the end-product of reaction. Besides, CP can oxidize biogenic (epinephrine, serotonin) and synthetic (*p*-phenylenediamine (*p*-PD), *o*-dianisidine) amines [3]. It also oxidizes glutathione in the presence of either NO [4] or $H_2O_2$ [5].

Hereditary deficiencies connected with inhibited synthesis or altered activity of CP (e.g. aceruloplasminemia in humans or knock-out of *CP* gene in mice) result in neurodegeneration and *diabetes mellitus*, both caused by oxidative stress due to accumulation of Fe (II) [6,7]. CP is a marker of inflammation in the acute phase, possessing the features of a universal antioxidant [8,9].

Correspondence: Alexej V. Sokolov, Department of Molecular Genetics of the Institute for Experimental Medicine of the Russian Academy of Medical Sciences, Acad. Pavlov street 12, Saint-Petersburg 197376, Russia. Tel: +7 812 23456006. Fax: +7 812 2349489. Email: biochem@nm.ru

ISSN 1071-5762 print/ISSN 1029-2470 online 
DOI: 10.1080/10715760802566574

Myeloperoxidase (MPO; EC 1.11.1.7, $M \sim 145$ kDa) is the heme-containing dimeric peroxidase of leukocytes [10,11]. This enzyme provides antimicrobial protection of an organism as it catalyses the formation of highly reactive species in reaction of halogenide ions with hydrogen peroxide [12]. Along with antimicrobial properties, MPO was shown to play a role in tumour suppression. These features of MPO explain frequent candidoses and increased risk of cancer in humans with hereditary deficiency of this enzyme [13,14].

It should be noticed that the antimicrobial effect of MPO may entail adverse cytotoxic effects. Pro-oxidants generated due to the presence of MPO can directly damage lipids [15,16], carbohydrates [17], proteins [18,19] and nucleic acids [20], which would provoke cell death [21].

MPO and the products of reactions catalysed by this enzyme were revealed in atherosclerotic plaques [22,23] and in respiratory tract secretions of patients with cystic fibrosis and asthma [24]. The risk of cardiovascular diseases becomes higher when pro-oxidants are generated by MPO oxidize apo-B and apo-AI lipoproteins [25,26].

In the late 1980s, Lyzlova's group published a series of papers [19,21,27] demonstrating that CP eliminated reactive oxygen species produced by MPO better than other serum anti-oxidants do. Those observations went almost unnoticed. Hence, when in 1991 a 'protein inhibitor with *M* about 150 kDa' was isolated from blood serum it was not identified [28]. Six years later it was shown that pro-oxidant activity of MPO is quenched upon its interaction with CP [29]. The authors even believed that binding to MPO and following its inhibition may be the major manifestation of CP antioxidant properties, however no effect of MPO upon oxidase activity of CP towards $Fe^{2+}$ and *o*-dianisidine was found [30]. Physiological significance of the interaction of CP with MPO was confirmed by Griffin et al. [31]. They found that in the case of systemic vasculites auto-antibodies to MPO preclude the inhibitory effect of CP causing an aggravation of the disease.

A large fraction of circulating MPO may be bound to CP. Indeed, when by means of affinity chromatography on Heparin-Sepharose, we isolated the CP-MPO complex from a CP preparation purified from human plasma [32], the content of MPO in the complex corresponded to all the MPO that could be isolated from the plasma used in our study. The latter phenomenon seems quite natural on account that the amount of plasma CP exceeds the entire amount of MPO in neutrophils of peripheral blood by two orders [33].

Apart from the CP-MPO complex, our studies of *in vitro* interaction of CP with proteins of neutrophils revealed complexes of CP with lactoferrin (LF) and serprocidins (elastase, cathepsin G, proteinase 3 and azurocidin) [32]. Furthermore a triple complex CP-LF-MPO was detected [34]. We found the CP-MPO, CP-LF and CP-LF-MPO complexes in biological fluids (serum, exudates) obtained from patients with inflammatory diseases [34]. Affinity of those proteins towards CP is likely to be a part of the general mechanism that neutralizes or modulates the activity of potentially toxic proteins released into blood from neutrophilic granules.

We describe here the enzymological aspects of CP-MPO interaction and the inhibitory effect of MPO upon proteolysis of CP. A presumable area of protein–protein interaction is discussed.

## Materials and methods

### *Chemicals*

The following reagents of analytical grade were used: Chelex 100 and molecular weight coloured markers (BioRad, USA); N-benzoyl-L-tyrosine ethyl ether, 2,2′-azino-di(3-ethylbenzthiazolinesulphonic acid) disodium salt (ABTS) and *p*-nitrophenol-t-Ala (ICN, USA); hydrogen peroxide, acetonitrile, dichloroethane, triethylamine, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, potassium cyanide and ethylenediaminetetraacetic acid (Merck, Germany); Sepharose 4B, Sepharose 6B, DEAE-Sephadex A-50, QAE-Sephadex A-50, CM-Sepharose and phenyl-Sepharose (Pharmacia, Sweden); sodium azide, guaiacol, glycerol, catalase, Coomassie R-250, 2-mercaptoethanol, ammonium persulphate, sucrose and Tris (Serva, Germany); aprotinin, bovine lactoperoxidase, *o*-dianisidine, dihydroxyphenylalanine, glycine, horseradish peroxidase, sodium dodecylsulphate (SDS), plasmin (3 units/mg protein), salmon protamine, Folin-Ciocalteu's reagent; taurine, tetramethylbenzidine, trypsin (10 000 units/mg protein), Triton X-100, *p*-phenylenediamine dihydrochloride (*p*-PD) and phenylmethylsulphonyl fluoride (Sigma, USA); acrylamide, arginine, N′,N′-methylene-bis-acrylamide and N,N,N′,N′-tetramethylethylene diamine (Laboratory Medigen, Russia).

### *Spectrophotometrical measurements*

Spectrophotometrical measurements were performed at room temperature using a spectrophotometer SF 2000-02 ('OKB Spectr', Russia). Mean square deviation in cases of linear regression of experimental data did not exceed 3%; differences among three parallel samples did not exceed 2%. Optical spectra of MPO (3 μM), CP (7.4 μM) and a mixture of proteins were measured in 50 mM Na-phosphate buffer, pH 7.4. Differences among three parallel samples did not exceed 1%. In control experiments we used a 3 μM solution of horseradish peroxidase or bovine lactoperoxidase.

*Enzyme isolation*

Monomeric CP stable at storage was obtained by affinity chromatography on protamine-Sepharose. Preparation had $A_{610}/A_{280} > 0.049$ and for more than 95% consisted of non-fragmented protein [35]. The same isolation protocol was used to obtain a preparation of CP without chromatography on heparin-Sepharose and without proteolysis inhibitors added to plasma. That protein would degrade upon storage at 4°C. Immobilization of $NH_2$-group-containing substances (protamine, arginine, heparin and aprotinin) on Sepharose was achieved by activation of the resin with cyanogen bromide, obtained by bromination of potassium cyanide [35].

MPO was purified from human leukocytes of healthy volunteers' blood by affinity chromatography on heparin-Sepharose, hydrophobic chromatography on phenyl-Sepharose and gel filtration, which yielded $A_{430}/A_{280}$ $(Rz) = 0.85$ [34].

Elastase was isolated from precipitate of leukocytes (20 g) that was suspended in 50 ml of 1 M NaCl, 50 mm Na-acetate, pH 4.7 and frozen/thawed with thrice-repeated 30-s sonication (44 Hz) interrupted by 60-s cooling on ice. Leukocyte extract obtained was centrifuged for 30 min at 15 000 g and 4° C. Supernatant was subjected to gel filtration on a Sephadex G-150 Superfine column ($5 \times 100$ cm) equilibrated with 1 M NaCl in 50 mm sodium-acetated buffer, pH 4.7. Fractions displaying elastase activity [36] were loaded on a column with aprotinin-Sepharose ($5 \times 10$ cm) after adding 1 M Tris to achieve pH 7.0. The column was washed with 1 m NaCl in 50 mM Tris-acetate, pH 7.4 to obtain $A_{280} <$ 0.005 in the effluent. Elastase was eluted with 200 mm sodium-acetate buffer, pH 4.3, after which the protein was purified from cathepsin G traces [37] using ion-exchange chromatography on a CM-cellulose column ($1 \times 5$ cm) equilibrated with 50 mm sodium-acetate buffer, pH 4.7. The protein diluted to the final buffer concentration 50 mM was loaded on the column, after which it was eluted with 100 ml of linear gradient $0 \rightarrow 1$ M NaCl in 50 mm sodium-acetate buffer, pH 4.7. Cathepsin-free elastase was obtained under conditions described above. Thus obtained elastase had specific activity 451 μcatal per mg protein as judged by *p*-nitrophenol-t-Ala hydrolysis.

*Limited proteolysis*

Limited proteolysis was carried out by incubation of CP, MPO and the equimolar mixture of these two proteins with serine proteinases at 37°C. Trypsin, elastase and plasmin were added as 1/1000, 1/50 and 1/100 (w/w) part of the proteins cleaved. Samples were taken after 5, 15 and 45 min of incubation, boiled with sample buffer for SDS/PAGE with 2-mercaptoethanol and run through SDS-containing 10% polyacrylamide gel [38].

*Myeloperoxidase and ceruloplasmin activity*

Such chromogenic substrates of MPO as guaiacol, orcinol, *o*-dianisidine, 4-chloro-1-naphtol, 3,3′,5,5′-tetramethylbenzidine and 2,2′-azino-di(3-ethyl-benzthiazolinesulphonic acid) disodium salt (ABTS) were used. Reaction mixture contained 100 μM $H_2O_2$, 3 nM MPO in 50 mM sodium-phosphate buffer, pH 7.4, and a substrate at final concentration of 500 μM. Upon adding $H_2O_2$ to the mixture the reaction rate was measured as $\triangle A_x$/min, where $x$ for guaiacol, orcinol and *o*-dianisidine is 450 nm, that for 4-chloro-1-naphtol is 600 nm, 650 nm for 3,3′5,5′-tetramethylbenzidine, and 414 nm for ABTS. Reaction rates in the presence of various amounts of CP were compared. In all our experiments reaction rates were obtained as $\triangle A_x$/min calculated by the pre-programmed spectrophotometer SF-2000-02 ('OKB Spectr', Russia). This ratio is based on the linear dependence of $A_x$ upon time in course of measurements. The linearity was confirmed by coefficients of determination for approximating straight lines that all were above 0.98.

To study an effect of CP on oxidation of ABTS by MPO, lactoperoxidase and horseradish peroxidase [39] we used a mixture of 10 nM peroxidase, 100 μM $H_2O_2$ and 1 mM ABTS in 0.1 M sodium-acetate buffer, pH 5.6 and various amounts of CP. The reaction was triggered by adding $H_2O_2$ to the mixture and its rate at room temperature was measured as $\triangle A_{414}$/min.

To study kinetics of the reaction either ABTS or $H_2O_2$ concentrations were varied. In the first case the reaction mixture contained 10 nM MPO, 80 μM $H_2O_2$ and 50–400 μM ABTS in 0.1 M sodium-acetate buffer, pH 5.6. In the second case the reaction mixture contained 4.5 nM MPO, 1 mm ABTS and 25–100 μM $H_2O_2$ in the same buffer. In order to study the effect of CP the latter was added to the mixture in needed amounts. The reaction was triggered by adding $H_2O_2$ to the mixture and its rate at room temperature was measured as $\triangle A_{414}$/min. The results were plotted in Hanes-Woolf coordinates (coefficient of determination value was higher than 0.99 for linear graphs). The plots were used to assay $K_m$ and $V_{max}$ for MPO in the presence of CP and without it.

Chlorinating activity of MPO was evaluated by measuring accumulation of taurine chloramine, the end-product in reaction of taurine with HOCl produced by MPO [30]. The reaction mixture contained 100 mM NaCl, 80 μM $H_2O_2$, 4 nM MPO, 10 mM taurine in 10 mM sodium-phosphate buffer, pH 7.4. Upon adding $H_2O_2$ to the mixture, reaction was allowed to run for 5 min at room temperature, after

which it was stopped by adding catalase (to 5 nM) and, after another 30 s, $NaN_3$ (to 4 μM). Taurine chloramine was assayed after adding 1 mM thio-(2-nitrobenzoic acid) by measuring $A_{414}$ proportional to the amount of HOCl produced.

CP oxidizes *p*-PD to a coloured product absorbing at 530 nM. Reaction mixture contained 100 mM sodium-acetate buffer, pH 5.5, 1.58 μM CP, *p*-PD as 0.25, 0.5, 0.75, 1 and 1.5 mM solution and an amount of MPO needed to achieve a required molar excess with respect to CP concentration. Reaction rate was assayed as $\triangle A_{530}$/min.

Kinetics of ferroxidase reaction was studied by measuring the rate of $Fe^{3+}$ formation. The method is based on spectrophotometry of a complex ($\lambda_{max}$ = 270–320 nm) that $Fe^{3+}$ forms with dimethylformamide. In control experiments we discovered that dimethylformamide does not alter the ferroxidase activity of Cp. Reaction mixture contained 0.1 m sodium-acetate buffer, pH 5.5, with dimethylformamide added to achieve 1%. More salt ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) was added in 30 μM thiourea to provide concentrations of 80, 100, 125, 150, 175 and 200 μM, and 0.79 μM CP. The reaction rate was measured as $\triangle A_{310}$/min.

o-dianisidine oxidation by Cp was studied in a solution containing 0.36 μM Cp in 0.1 M sodium-acetate buffer, pH 5.5, and o-dianisidine dihydrochloride added as a portion of its 2 mM stock solution in 1% Triton X-100 to provide the resulting concentrations of 0.1, 0.2 and 0.4 mM. The reaction rate was measured as $\triangle A_{450}$/min.

Oxidation of dihydroxyphenylalanine by Cp was followed by absorption at 320 nm ($\triangle A_{320}$/min). Reaction mixture contained 0.1 M sodium-acetate buffer, pH 5.5, 1.58 μM Cp and 0.1, 0.25, 0.5 or 1.0 mM dihydroxyphenylalanine. A Hanes-Woolf plot was drawn (coefficient of determination value for linear graphs was higher than 0.98) and $K_m$ and $V_{max}$ were assayed for CP both with and without MPO.

*Statistical data manipulation*

Experiments were repeated three times ($n = 3$) and the mean values were calculated as $X_m = (1/n)\sigma X_i$, where $X_i$ is the value of each following sample. The standard error was expressed as

$$S^*/n, \text{ where } S^* = \sqrt{\frac{\sum(X_i - X_m)^2}{(n-1)}}.$$

The confidence interval was calculated as $X_m \pm (S^*/n^{1/2})t_{n-1,1-\alpha/2}$, for which $t$ was found in the table of values on condition that in our experiments $\alpha = 0.05$.

## Results and discussion

*Effect of CP upon peroxidase and chlorinating activity of MPO*

The inhibitory capacity of CP depends on the size of aromatic substrate of MPO. Figure 1 shows how CP concentration affects MPO peroxidase activity towards six various aromatic substrates: guaiacol, orcinol, *o*-dianisidine, 4-chloro-1-naphtol, tetramethylbenzidine and ABTS. One can see that CP only slightly inhibits the oxidation of small aromatic substrates such as guaiacol and orcinol. Upon adding some noticeable excess of CP (0.54 μM) the relative velocity of the reaction dropped only to 60%. Inhibition of MPO peroxidase activity towards larger substrates goes much more efficiently, e.g. the rate of oxidizing of *o*-dianisidine and 4-chloro-1-naphtol was decreased by 0.54 μM CP to 40% and that of tetramethylbenzidine to 0. Oxidation of the largest of all studied MPO substrates, ABTS, was already

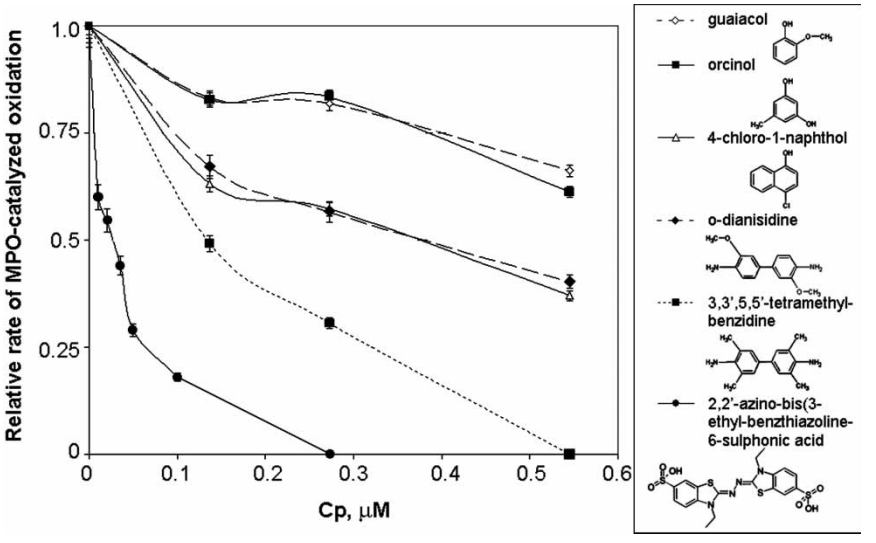


Figure 1. Effect of CP upon the relative rate of oxidation of various aromatic substrates by MPO. The reaction mixture would contain 100 μM $H_2O_2$, 3 nM MPO in 50 mM sodium-phosphate buffer, pH 7.4, and a substrate at final concentration 500 μM.

completely blocked by 0.27 μM CP. Therefore, the larger an aromatic substrate is, the more pronounced is the capacity of CP to inhibit their oxidation in the active centre of MPO. The interaction of CP and MPO is likely to entail a steric barrier that precludes binding of aromatic substrates to MPO. A steric barrier is stronger for larger substrates and, therefore, their oxidation is more affected by CP.

Although some of the substances used are the substrates of CP (*o*-dianisidine, orcinol, guaiacol), the enzyme did not catalyse their oxidation at neutral pH, as demonstrated in our control experiments. Neither did CP inhibit the oxidation of aromatic substrates by horseradish peroxidase or bovine lactoperoxidase in control series (Figure 2). This is more indicative of rather a specific interaction of CP with MPO than of its effect upon the substrates. Inhibition of ABTS oxidation ran more efficiently at pH 7.4 than at pH 5.6, which seems to result from the pH-dependent interaction between CP and MPO. We have shown previously [34] that affinity of CP towards MPO goes down upon pH decrease.

To assay the kinetics parameters of the peroxidase reaction catalysed by MPO in the presence and in the absence of CP we estimated the reaction rate as a function of substrate concentration ($H_2O_2$ and ABTS). The plot in Hanes-Woolf coordinates (S, S/V) illustrates the data obtained upon varying of $H_2O_2$ concentration from 25 to 100 μM (Figure 3). Peroxidase reaction in the absence of CP had $K_m = 37.9 \pm 0.5$ μM and $V_{max} = 0.46 \pm 0.01$ $\triangle A_{414}$/min (Figure 3, line 1). Adding 0.25 μm CP (which provides 1MPO:55CP molar relation) virtually does not affect the Michaelis constant, though the reaction maximum velocity $V'_{max}$ becomes 1.5-times lower and is equal to $0.31 \pm 0.01$ $\triangle A_{414}$/min (Figure 3, line 2). This change of kinetics suggests that CP inhibits the peroxidase reaction, but does not compete with MPO for hydrogen peroxide. The inhibition constant, calculated as:

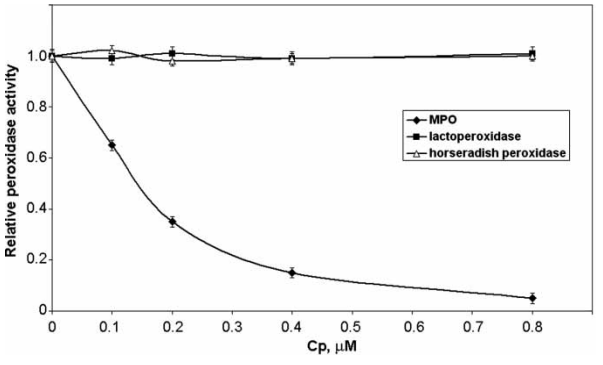


Figure 2. Effect of CP upon the relative rate of oxidation of ABTS by MPO, lactoperoxidase and horseradish peroxidase. The reaction mixture contained 10 nM of peroxidase, 1 mM ABTS and 100 μM $H_2O_2$ in 0.1 M sodium-acetate buffer, pH 5.6.

$$K_i = \frac{[CP]}{\frac{V_{max}}{V'_{max}}} - 1$$

was equal to $0.51 \pm 0.02$ μM. It is worth noting that the inhibitory effect was the property of non-proteolysed CP only (Figure 3, 1 in insert). In contrast, CP isolated without proteolysis inhibitors and, hence, degraded upon storage (preparation contained no molecules with $M = 132$ kDa) did not inhibit the peroxidase activity of MPO (Figure 3, line 3; 2 in insert).

Similar measurements of the reaction parameters were done using various concentrations of ABTS (50–900 μm) in the absence of CP and in the presence of its 16- and 32-fold molar excess. The data plotted in Hanes-Woolf coordinates (S, S/V) are presented in Figure 4. It is seen that adding CP increases the Michaelis constant from $K_m = 360 \pm 5$ μM to $K'_m = 394 \pm 5$ μM (in case of 1 MPO:16 CP) and to $K''_m = 450 \pm 5$ μM (1 MPO:32 CP). At the same time the maximum velocity of the reaction remains virtually constant ($V_{max} = 0.46 \pm 0.01$ $\triangle A_{414}$/min). CP plays a role of a competitive inhibitor in this process, impeding ABTS binding in the active centre of MPO. The values of the inhibition constant calculated for various CP concentrations according to

$$K_i = \frac{[CP]}{\frac{K'_m}{K_m} - 1}$$

were the same and equal to $1.136 \pm 0.004$ μM.

The major cytotoxic derivatives originate as a result of chlorinating activity of MPO. Hence, the capacity of CP to alter this activity was studied along with the inhibitory effect of CP on the peroxidase reaction. Under the conditions of our experiment 0.5 μM CP decreased the chlorinating activity of MPO by 60% (Figure 5, dashed line, 1). Inhibition of MPO activity cannot be ascribed merely to interception of HOCl by reactive amino acid residues in CP molecule. Indeed the amino acid content of intact CP is identical to that of the partially proteolysed protein, as shown previously [40]. However, proteolysed CP (preparation contained no 132 kDa molecules) had a negligibly small effect on the chlorinating activity of MPO (Figure 5, solid line, 2). Previously we obtained similar results when studying the effect of CP on MPO activity assayed by luminol chemiluminescence in reaction with HOCl [41]. It appeared that only non-proteolysed CP is able to inhibit MPO.

Our results show that both peroxidase and chlorinating activity of MPO are suppressed exclusively by non-proteolysed CP, which is in agreement with earlier data [29]. Segelmark et al. [29] have reported that effect of CP upon the MPO peroxidase activity decreases in course of its purification from blood

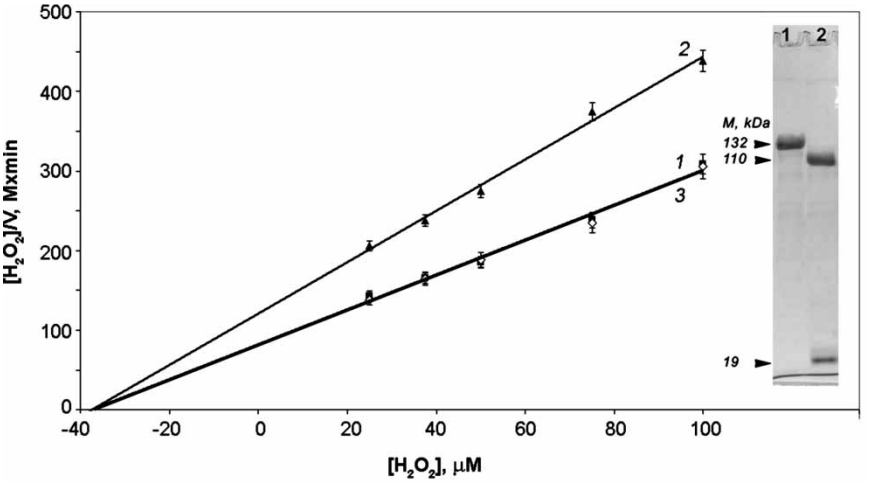


Figure 3. Hanes-Woolf plot for peroxidase reaction catalysed solely by MPO (1) and by MPO in the presence of intact (2) and partially proteolysed (3) CP (0.25 μM). The reaction mixture contained 4.5 nM MPO, 1 mM ABTS and 25–100 μM $H_2O_2$ in 0.1 M sodium-acetate buffer, pH 5.6. In the insert: SDS/PAGE of intact (1) and proteolysed CP (2).



plasma and in the subsequent storage. It is also known that CP purified without protease inhibitors gradually becomes partly proteolysed [42]. We have shown previously by disc-electrophoresis and affinity chromatography that limited proteolysis of CP does not impede forming a complex CP-MPO [34]. It seems likely that limited proteolysis affects the site(s) of no crucial importance for the binding of CP with MPO, though it is a prerequisite for modification of the latter's activity.

*Effect of MPO on the limited hydrolysis of CP by serine proteinases*

A possible explanation for our data that integrity of CP is essential for its inhibitory effect upon MPO could be the contact of one of proteinase-vulnerable loops of CP with the entrance to the heme pocket in MPO. In this case MPO perhaps can prevent the cleavage of CP by trypsin, elastase and plasmin. The above-mentioned proteinases differ by the specificity and velocity of hydrolysis demonstrated towards CP. Trypsin and plasmin with high and low intensity, respectively, cut peptide bonds in CP formed by lysyl and arginyl. Elastase cleaves peptide bonds with N-terminal hydrophobic amino acids (V, A, etc.), the efficiency of proteolysis being as low as in the case of plasmin. In all cases the limited proteolysis of CP by these proteinases starts with the first splitting of its polypeptide chain between domains 5 and 6 (trypsin and plasmin cut after $^{887}$R, while elastase cuts after $^{888}$V) [43]. This cleavage results in occurrence of CP fragments with $M = 110$ and 19 kDa clearly visualized by SDS/PAGE.

For experimental convenience we estimated the amounts of each proteinase needed to hydrolyse CP with similar efficiency within certain time intervals. MPO, in contrast to CP, was not cleaved by proteinases used in our control experiments and was always resolved in electrophoresis into two intact bands with $M = 62$ and 12 kDa corresponding to $\alpha$- and $\beta$-chains of the enzyme.

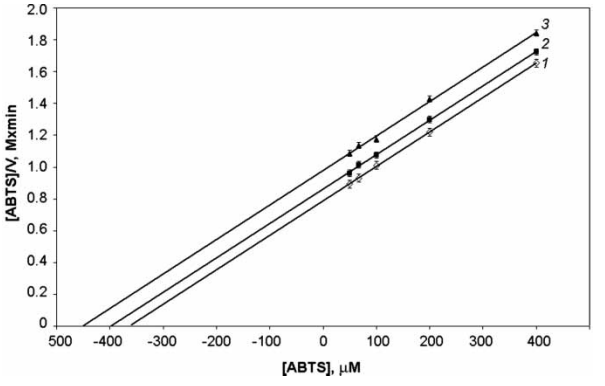


Figure 4. Hanes-Woolf plot illustrating inhibitory effect of CP upon MPO. The reaction mixture contained 10 nM MPO, 80 μM $H_2O_2$ and 50–400 μM ABTS in 0.1 M sodium-acetate buffer, pH 5.6. Data were collected in the absence of CP (1), with 0.16 μM (2) and 0.32 μM (3) of CP.

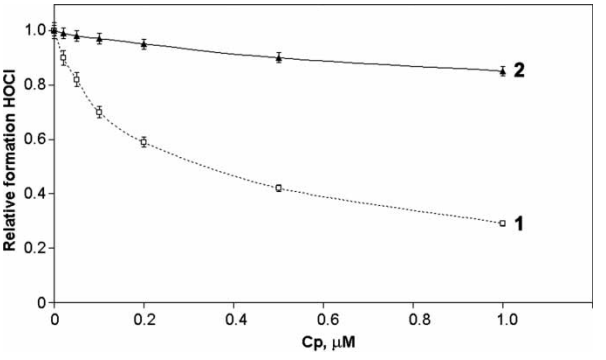


Figure 5. Chlorinating activity of MPO in the presence of proteolysed (solid line, 2) and intact (dashed line, 1) CP. The reaction mixture contained 100 mM NaCl, 80 μM $H_2O_2$, 4 nM MPO, 10 mm taurine in 10 mM sodium-phosphate buffer, pH 7.4.

Under trypsin treatment the amount of protein in the initially prevailing 132 kDa-band decreased with time (Figure 6A: 1–5). The fragment with $M = 110$ kDa did not accumulate, since hydrolysis of other peptide bonds proceeded simultaneously. However, splitting of the bond(s) between domains 5 and 6 is evidenced by an increasing amount of the 19 kDa-fragment. An increase of the amount of fragments with $M = 110$ and 19 kDa was hardly noticeable in the presence of MPO. However, judging by the appearance of fragments with $M = 90$–80 kDa, the hydrolysis of other bonds in CP went on (Figure 6A: 6–9).

Proteolysis of CP with elastase and plasmin in the presence of MPO was hampered not only at the site between domains 5 and 6, but also at other sites susceptible to proteolytic attack (Figure 6B and C). When CP was treated with elastase fragments with $M = 20$–80 kDa it had appeared, while the 132 kDa-band corresponding to non-degraded CP had virtually disappeared after 45 min of hydrolysis. However, when MPO was added to the mixture, about half of the 132 kDa-band remained and only the fragments with $M$ higher than 80 kDa had appeared. Plasmin could hardly hydrolyse CP in the presence of MPO. The latter retained its two non-proteolysed chains revealed as bands with $M = 62$ and 12 kDa, as mentioned above (see Figure 6).

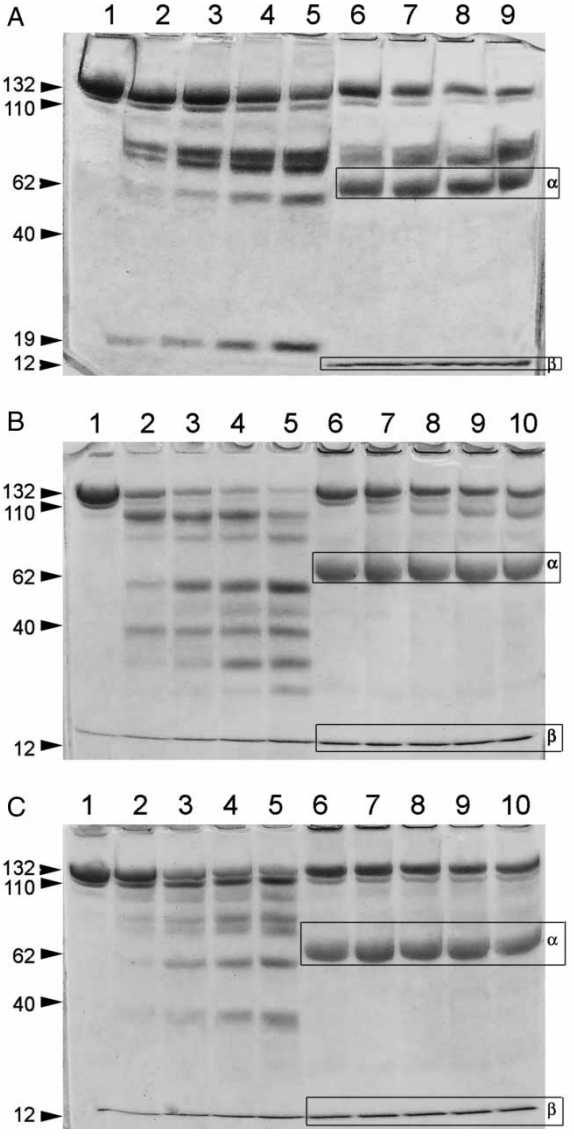


Figure 6. SDS/PAGE of CP (20 μg; lanes 1–5) and the CP-MPO complex (20 μg of each protein; lanes 6–10) treated with proteinases. (A) 1, intact CP; 2–5, CP-MPO complex; 6–9, 5, 15, 30 and 45 min treatment with trypsin. (B) 1–5 and 6–10: 0, 5, 15, 30 and 45 min treatment with elastase. (C) 1–5 and 6–10: 0, 5, 15, 30 and 45 min treatment with plasmin. Arrows mark $M$ (kDa), The frame mark bands corresponded $\alpha$- and $\beta$-chains of MPO.

Thus, in our experiments MPO protected CP against the attack by serine proteinases of the loop linking domains 5 and 6. This protection possibly appears as the contact of CP with MPO makes inaccessible the peptide stretch in CP containing the bonds susceptible to proteolysis. Inhibition of proteolytic cleavage of the bonds beyond the region of protein–protein interaction might be explained by the trigger effect: the proteinases used hydrolyse peptide bonds in CP in certain order and, unless the first one is cleaved, the splitting of others is hindered.

The observed competitive mode of inhibition by CP of oxidation aromatic substrates by MPO and reciprocal phenomenon of the protection of the loop linking domains 5 and 6 in CP (a.a. 885–892) from proteinases enable us to suggest an involvement of this loop of CP in the protein–protein interactions near MPO active site. This conclusion has been confirmed by analysis of the X-ray structure of CP-MPO complex (in press).

### *Spectral studies of MPO in complex with CP*

The absorption spectrum of MPO is characterized by an intense Soret peak at 430 nm (Figure 7, curve 1). There are no specific features in CP spectrum near this wavelength (curve 2). In our experiments, adding CP to MPO resulted in a shift of the Soret peak maximum from 430 to 427 nm (curve 3). This shift is not found in an arithmetic sum of CP and MPO spectra. Lactoperoxidase and horseradish peroxidase (also heme-containing) are characterized by an intense Soret peak at 413 and 403 nm, respectively. Differences in absorption maxima are explained by peculiar configurations of heme in each case. While heme in horseradish peroxidase is a planar structure, it is slightly bent in lactoperoxidase and in MPO this bend is the most pronounced [44]. In a control experiment when CP was added to lactoperoxidase or horseradish peroxidase the shift of absorption maximum has not appeared. It can be concluded that formation of the CP-MPO complex results in an alteration of heme conformation.

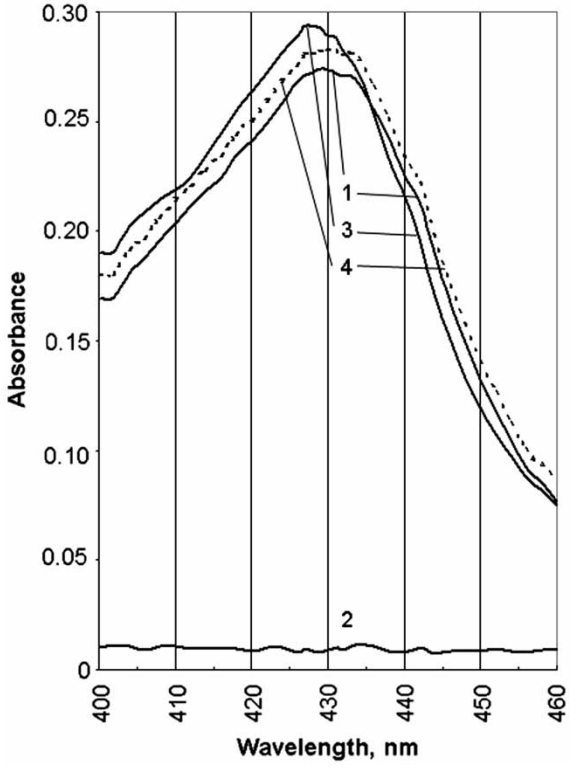


Figure 7. Optical spectra of 3 μM MPO (1), 7.4 μM CP (2) and 7.4 μM CP plus 3 μM MPO (3), dashed line shows the arithmetic sum of CP and MPO spectra (4).

*Effect of MPO upon the oxidase activity of CP*

It was considered previously that enzymatic and immunogenic properties of CP remain unaltered in the course of its interaction with MPO [30]. Like Park et al. [30], we observed no effect of MPO on the oxidase activity of CP performed with $Fe^{2+}$ and *o*-dianisidine. This means that neither $K_m$ nor the rate of reactions both with $Fe^{2+}$ ($K_m = 46.5 \pm 1.9$ μM, $K'_m = 45.6 \pm 1.7$ μM, $V_{max} = 0.918 \pm 0.020$ $\triangle A_{310}$/h, $V'_{max} = 0.930 \pm 0.028$ $\triangle A_{310}$/h) and *o*-dianisidine ($K_m = 155 \pm 5$ μM, $K'_m = 148 \pm 9$ μM, $V_{max} = 0.834 \pm 0.042$ $\triangle A_{450}$/h, $V'_{max} = 0.840 \pm 0.038$ $\triangle A_{450}$/h) were changed. Similarly, in our experiments the rate of dihydroxyphenylalanine oxidation by CP did not change when MPO was added to reaction mixture ($K_m = 99 \pm 4$ μm, $K'_m = 105 \pm 9$ μm, $V_{max} = 0.396 \pm 0.004$ $\triangle A_{320}$/h, $V'_{max} = 0.401 \pm 0.006$ $\triangle A_{320}$/h).

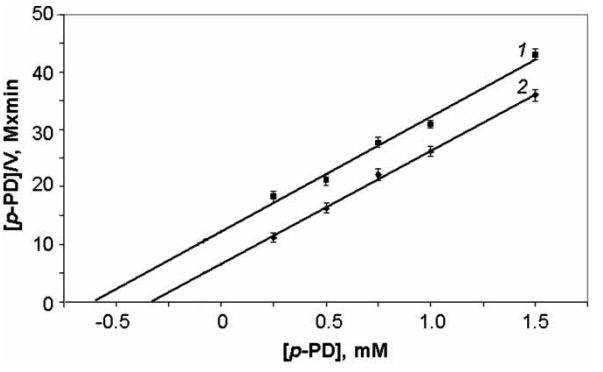


Figure 8. Hanes-Woolf plot illustrating oxidation of *p*-PD catalysed by CP (1) and by CP in the presence of 0.5-molar excess of MPO (2). The reaction mixture contained 100 mM sodium-acetate buffer, pH 5.5, 1.58 μM CP, *p*-PD as 0.25–1.5 mM solution.

However, oxidation of *p*-phenylenediamine (*p*-PD) by CP was activated in the presence of MPO (Figure 8). Linear graph (1) depicts in Hanes-Woolf coordinates the oxidase activity of CP in reaction with *p*-PD in the absence of MPO is parallel to analogous graph (2) plotted in the presence of MPO (1 MPO:2 CP). Interaction of CP with MPO virtually did not affect the reaction velocity ($V_{max} = 3.00 \pm 0.09$ $\triangle A_{530}$/h, $V'_{max} = 3.12 \pm 0.09$ $\triangle A_{530}$/h), but caused a 1.85 time decrease of the Michaelis constant ($K_m = 612 \pm 12$ μM, $K'_m = 330 \pm 16$ μM), which is indicative of the associative type of activation. The activation constant calculated according to

$$K_a = \frac{[MPO]}{\frac{K_m}{K'_m} - 1}$$

was equal to $0.203 \pm 0.008$ μM. The activating effect of MPO that was observed at a ratio of proteins 1 MPO:2 CP did not change upon increasing the amount of MPO. In control experiments *p*-PD was not oxidized by MPO under the same conditions.

The ability of MPO to modify the CP-catalysed oxidation of *p*-PD, but not of other substrates studied ($Fe^{2+}$, *o*-dianisidine, dihydroxyphenylalanine), may be explained by the contact of MPO with CP in close proximity to the *p*-PD-binding site. This binding site in the CP molecule is revealed by crystallography studies and includes amino acid residues $^{667}$H and $^{669}$W [45]. It seems likely that other substrates from the group of synthetic aromatic amines bind to CP in different sites, as judged by the results obtained in the present study.

The inhibition constants calculated in our study ($\approx 0.5$–1 μM) correspond to a dissociation constant ($K_d$) for the complex composed of an enzyme (MPO) and an inhibitor (CP). Similarly, the activation constant ($\approx 0.2$ μM) represents the $K_d$ for the complex of the enzyme (CP) with the activator (MPO). Previously the $K_d$ for the CP-MPO complex was measured by ELISA [31]. The authors used conditions very different from those in our experiments (neutral pH, albumin in the mixture). Nevertheless, their $K_d = 0.134$ μM is very close to ours. Such an agreement of parameters obtained under such different experimental conditions is likely to reflect the relative stability of the CP-MPO complex in varying environments (e.g. pH and ionic strength).

Summarizing our data one can suggest that in our study MPO protected the anti-oxidant potential of

CP, which is particularly important in foci of inflammation where numerous serine proteinases can be found. Our previous study showed that the CP-MPO complex dissociates at pH lower than 3.9 [34]. Such a tolerance of the complex to low pH seems to be conditioned by acidic environment in inflammation foci.

HOCl produced *in vitro* by MPO brings forth hydroxyl radicals in reaction with $Fe^{2+}$ [46]. However, if a similar reaction occurs *in vivo*, the ferroxidase activity of CP is likely to prevent the contact of Fe (II) with MPO derivatives. Such a notion seems interesting on account that the CP-MPO complex was revealed in blood serum and exudates obtained from patients with inflammatory and purulent processes. It seems even more important as the presence of neutrophil-derived lactoferrin that chelates $Fe^{3+}$ and forms a triple complex with CP and MPO was documented in a study of the same biological fluids [33].

Thus, it can be suggested that interaction of CP with MPO diminishes the oxidative stress occurring in the course of inflammatory reactions. Inhibition and activation constants obtained in our study reflect the affinity of CP towards MPO (up to 1 μM). Consequently, a 2–3-fold increase of CP content in serum upon inflammation (from 3 μM to 6–9 μM) [47] should be regarded as sort of insurance of an organism against the destruction of its own molecules by endogenous MPO of leukocytes.



## Acknowledgements

This study was supported by RFBR grants 06-04-48602, 08-04-00532 and the program of Basic Research of the Russian Academy of Sciences Presidium 'Basic sciences for Medicine'. The authors are grateful to Professor V. N. Kokryakov and Professor M. M. Shavlovski (Institute of Experimental Medicine, St. Petersburg) for granted materials, valuable advice and constructive discussions.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References

[1] Osaki S, Johnson DA, Frieden E. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J Biol Chem 1966;241:2746–2751.

[2] Stoj C, Kosman DJ. Cuprous oxidase activity of yeast Fet3p and human ceruloplasmin: implication for function. FEBS Lett 2003;554:422–426.

[3] Young SN, Curzon G. A method for obtaining linear reciprocal plots with caeruloplasmin and its application in a study of the kinetic parameters of caeruloplasmin substrates. Biochem J 1972;129:273–283.

[4] Inoue K, Akaike T, Miyamoto Y, Okamoto T, Sawa T, Otagiri M, Suzuki S, Yoshimura T, Maeda H. Nitrosothiol formation catalyzed by ceruloplasmin. Implication for cytoprotective mechanism *in vivo*. J Biol Chem 1999;274:27069–27075.

[5] Kim IG, Park SY. Requirement of intact human ceruloplasmin for the glutathione-linked peroxidase activity. FEBS Lett 1998;437:293–296.

[6] Harris ZL, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RTA, Gitlin JD. Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. Proc Natl Acad Sci USA 1995;92:2539–2543.

[7] Vassiliev V, Harris ZL, Zatta P. Ceruloplasmin in neurodegenerative diseases. Brain Res Rev 2005;49:633–640.

[8] Goldstein IM, Kaplan HB, Edelson HS, Weissmann G. Ceruloplasmin: an acute phase reactant that scavenges oxygen-derived free radicals. Ann NY Acad Sci 1982;389:368–379.

[9] Atanasiu RL, Stea D, Mateescu MA, Vergely C, Dalloz F, Briot F, Maupoil V, Nadeau R, Rochette L. Direct evidence of caeruloplasmin antioxidant properties. Mol Cell Biochem 1998;189:127–135.

[10] Agner K. Verdoperoxidase. A ferment isolated from leukocytes. Acta Physiol Scand 1941;A2(Suppl. 8):1–62.

[11] Blair-Johnson M, Fiedler T, Fenna R. Human myeloperoxidase: structure of a cyanide complex and its interaction with bromide and thiocyanate substrates at 1.9 Å resolution. Biochemistry 2001;4:13990–13997.

[12] Klebanoff SJ. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. J Bacteriol 1968;95:2131–2138.

[13] Lanza F. Clinical manifestation of myeloperoxidase deficiency. J Mol Med 1998;76:676–681.

[14] Aratani Y, Koyama H, Nyui S, Suzuki K, Kura F, Maeda N. Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. Infect Immun 1999;67:1828–1836.

[15] Arnhold J, Osipov AN, Spalteholz H, Panasenko OM, Schiller J. Effects of hypochlorous acid on unsaturated phosphatidylcholines. Free Radic Biol Med 2001;31:1111–1119.

[16] Panasenko OM, Spalteholz H, Schiller J, Arnhold J. Myeloperoxidase-induced formation of chlorohydrins and lysophospholipids from unsaturated phosphatidylcholines. Free Radic Biol Med 2003;34:553–562.

[17] Schiller J, Arnhold J, Gründer W, Arnold K. The action of hypochlorous acid on polymeric components of cartilage. Biol Chem Hoppe Seyler 1994;375:167–172.

[18] Aruoma OI, Halliwell B. Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. Biochem J 1987;248:973–976.

[19] Sharonov BP, Govorova NJU, Lyzlova SN. A comparative study of serum proteins ability to scavenge active oxygen species: O2- and OCl-. Biochem Int 1988;17:783–790.

[20] Hawkins CL, Davies MJ. Hypochlorite-induced damage to DNA, RNA, and polynucleotides: formation of chloramines and nitrogen-centered radicals. Chem Res Toxicol 2002;15:83–92.

[21] Govorova NIU, Sharonov BP, Lyzlova SN. Erythrocyte oxidative damage by myeloperoxidase. The protective action of serum proteins. Biull Eksp Biol Med 1989;107:428–430.

[22] Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest 1994;94:437–444.

[23] Hazell LJ, Arnold L, Flowers D, Waeg G, Malle E, Stocker R. Presence of hypochlorite-modified proteins in human atherosclerotic lesions. J Clin Invest 1996;97:1535–1544.

[24] Kettle AJ, Chan T, Osberg I, Senthilmohan R, Chapman AL, Mocatta TJ, Wagener JS. Myeloperoxidase and protein

oxidation in the airways of young children with cystic fibrosis. Am J Respir Crit Care Med 2004;170:1317–1323.
[25] Carr AC, Myzak MC, Stocker R, McCall MR, Frei B. Myeloperoxidase binds to low-density lipoprotein: potential implications for atherosclerosis. FEBS Lett 2000;487: 176–180.
[26] Zheng L, Nukuna B, Brennan M-L, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, Hazen SL. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. J Clin Invest 2004;114:529–541.
[27] Govorova NIU, Sharonov BP, Iankovskii OIU, Lyzlova SN. Inactivation of active forms of oxygen generated by myeloperoxidase and serum proteins. Dokl Akad Nauk SSSR 1986;290:480–483.
[28] Yea CM, Dularay B, Elson CJ. Identification of a myeloperoxidase inhibitor from normal human serum. Clin Exp Immunol 1991;84:347–352.
[29] Segelmark M, Persson B, Hellmark T, Wieslander J. Binding and inhibition of myeloperoxidase (MPO): a major function of ceruloplasmin? Clin Exp Immunol 1997;108:167–174.
[30] Park YS, Suzuki K, Mumby S, Taniguchi N, Gutteridge JMC. Antioxidant binding of caeruloplasmin with myeloperoxidase: myeloperoxidase is inhibited, but oxidase, peroxidase and and immunoreactive properties of caeruloplasmin remain intact. Free Radic Res 2000;33:261–265.
[31] Griffin SV, Chapman PT, Lianos EA, Lockwood CM. The inhibition of myeloperoxidase by ceruloplasmin can be reversed by anti-myeloperoxidase antibodies. Kidney Int 1999;55:917–925.
[32] Sokolov AV, Pulina MO, Ageeva KV, Runova OL, Zakharova ET, Vasilyev VB. Identification of leukocyte cationic proteins that interact with ceruloplasmin. Biochemistry (Moscow) 2007;72:872–877.
[33] Kokryakov VN. Biology of antibiotics of animal origin. St. Petersburg: Nauka; 1999.
[34] Sokolov AV, Pulina MO, Ageeva KV, Ayrapetov MI, Berlov MN, Volgin GN, Markov AG, Yablonsky PK, Kolodkin NI, Zakharova ET, Vasilyev VB. Interaction of ceruloplasmin, lactoferrin and myeloperoxidase. Biochemistry (Moscow) 2007;72:409–415.
[35] Sokolov AV, Zakharova ET, Shavlovskii MM, Vasil'ev VB. Isolation of stable human ceruloplasmin and its interaction with salmon protamine. Bioorg Khim 2005;31:269–279.
[36] Freinstein G, Janoff A. A rapid method of purification of human granulocyte cationic neutral proteases: purification and further characterization of human granulocyte elastase. Biochim Biophys Acta 1975;403:493–505.
[37] Atassi MZ, Manshouri T. Design of peptide enzymes (pepzymes): Surface-simulation synthetic peptides that mimic the chymotrypsin and trypsin active sites exhibit the activity and specificity of the respective enzyme. Proc Natl Acad Sci USA 1993;90:8282–8286.
[38] Laemmli UK. Cleavage of structural proteins during the assembly of the head of Bacteriophag. Nature (London) 1970;227:680–686.
[39] Reszka KJ, McCormick ML, Britigan BE. Peroxidase- and nitrite-dependent metabolism of the anthracycline anticancer agents daunorubicin and doxorubicin. Biochemistry 2001;40:15349–15361.
[40] Prozorovski VN, Rashkovetski LG, Shavlovski MM, Vasiliev VB, Neifakh SA. Evidence that human ceruloplasmin molecule consists of homologous parts. Int J Pept Protein Res 1982;19:40–53.
[41] Panasenko OM, Chekanov AV, Vlasova II, Sokolov AV, Ageeva KV, Pulina MO, Cherkalina OS, Vasilyev VB. A study of the effect of ceruloplasmin and lactoferrin on chlorination activity of leukocytic myeloperoxidase using chemiluminescence method. Biofizika 2008;53:573–581.
[42] Ehrenwald E, Fox PL. Isolation of nonlabile human ceruloplasmin by chromatographic removal of a plasma metalloproteinase. Arch Biochem Biophys 1994;309:392–395.
[43] Sang QA. Specific proteolysis of ceruloplasmin by leukocyte elastase. Biochem Mol Biol Int 1995;37:573–581.
[44] Furtmüller PG, Zederbauer M, Jantschko W, Helm J, Bogner M, Jakopitsch C, Obinger C. Active site structure and catalytic mechanisms of human peroxidases. Arch Biochem Biophys 2006;445:199–213.
[45] Zaitsev VN, Zaitseva I, Papiz M, Lindley PL. An X-ray crystallographic study of the binding center of azide inhibitor and organic substrates of ceruloplasmin, a multi-copper oxidase in plasma. J Biol Inorg Chem 1999;4:579–587.
[46] Candeias LP, Stratford MR, Wardman P. Formation of hydroxyl radicals on reaction of hypochlorous acid with ferrocyanide, a model iron (II) complex. Free Radic Res 1994;20:241–249.
[47] Gitlin JD. Transcriptional regulation of ceruloplasmin gene expression during inflammation. J Biol Chem 1988;263: 6281–6287.



This paper was first published online on iFirst on 28 November 2008.