MOLECULAR BIOPHYSICS

Influence of Ceruloplasmin and Lactoferrin on the Chlorination Activity of Leukocyte Myeloperoxidase Assayed by Chemiluminescence

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Abstract—We demonstrate that addition of H_2O_2 to a mixture of myeloperoxidase (MPO), chloride and luminol immediately evokes a short intense flash of chemiluminescence (CL). This flash is diminished in the absence of MPO or chloride, and in the complete system it is suppressed by an MPO inhibitor azide, hypochlorite scavengers taurine or methionine, or an MPO peroxidase-cycle substrate guaiacol. Hence, this CL is mostly due to the MPO halogenation function; a measure of this activity is provided by the integral CL. With three independent methods (CL, taurine chlorination, and peroxidase assay) it is shown that MPO activity is suppressed by ceruloplasmin (Cp). Lactoferrin has no effect either on MPO or on the MPO–Cp complex. It is also shown that peroxidase inhibition by Cp is the stronger the larger is the MPO substrate, which suggests steric hindrances to substrate binding in the MPO–Cp complex. Importantly, the conventional chlorination and peroxidase assays detect MPO inhibition by Cp only at a large excess of the latter, whereas the CL assay reveals it at stoichiometric ratios characteristic of the naturally occurring protein complexes.

Key words: myeloperoxidase, ceruloplasmin, hypochlorite, chemiluminescence, protein–protein interactions

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INTRODUCTION

Myeloperoxidase (MPO), ceruloplasmin (Cp), and lactoferrin (Lf) are metal-associated proteins found in human blood and directly involved in redox processes, innate immunity, and metal metabolism [1–3].

MPO, a cationic (pI 9–10) glycohemoprotein of 145 kDa, is present in leukocytes, mainly in the azurophilic granules of neutrophils, and is released upon degranulation of these cells in inflammation foci [1, 4]. This enzyme [donor: H_2O_2 oxidoreductase, EC 1.11.1.7] has peroxidase activity and can also oxidize halides (Cl^-, Br^-, I^-) as well as pseudohalide SCN^- to hypohalous acids [5].

A simplified scheme of MPO functions is outlined in Fig. 1. Interaction of the native ferric form of MPO with H_2O_2 (step *1*) gives a high-potential MPO compound I, which may then perform either two-electron oxidation of a halide (X) (step *2*) to close the halogenation cycle, or consecutive one-electron oxidation of a number of substances (AH), itself converting to com-

pound II (step *3*) and native state (step *4*) to close the peroxidase cycle [1, 4, 5].

Hypohalous acids are strong oxidants (especially HOCl and HOBr) and react with numerous biologically important substances: nucleic acids, carbohydrates, proteins, lipids, vitamins, antioxidants [6–10]. The consequences of these reactions, depending on MPO activ-
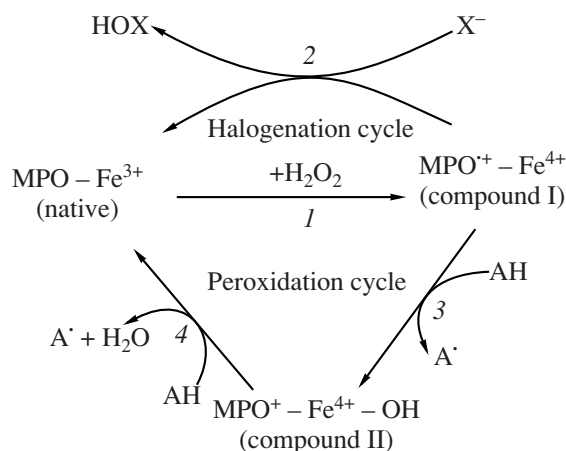

Fig. 1. Scheme of myeloperoxidase function.

ity and targeting of its products, may be both positive (anti-pathogen action) and negative (cytotoxicity, oxidative stress).

One of the means of regulating MPO activity in blood may be its binding with Cp, a 132-kDa metalloprotein of the α_2-globulin fraction. This enzyme [EC 1.16.3.1] has six tightly bound copper ions and exhibits ferroxidase, amine oxidase, peroxidase [11], and superoxide dismutase [12] activities, thus possessing both antioxidant [2, 13, 14] and prooxidant properties [15]. Under physiological conditions this acidic protein (pI 4.7) can make sturdy complexes with MPO [16]. Upon MPO–Cp binding, both the peroxidase [17, 18] and the halogenation [11] activities of MPO are inhibited while the antioxidant Cp functions are not affected [11].

We have recently shown [16] that the MPO–Cp complex may be joined by another cationic protein Lf (78 kDa, pI 8–9), which is contained in specific granules of neutrophils [19] and released upon degranulation, exhibiting immunomodulator and anti-inflammatory properties [20]. Furthermore, MPO–Cp and MPO–Cp–Lf complexes have been found in sera of patients with inflammations but not in healthy donors [16].

Here we examined the influence of Cp and Lf on MPO activity; along with conventional tests, we used a highly sensitive assay for chemiluminescence (CL) attending the reaction of hypochlorite with luminol [21–23]. In this way, we succeeded in detecting MPO inhibition by Cp at really stoichiometric ratios.

EXPERIMENTAL

Reagents. All buffer salts, HOCl, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), guaiacol (2-methoxyphenol), ABTS [sodium 2,2'-azinobis(3-ethyl-benzotriazoline-6-sulfonate)], catalase and horseradish peroxidase were from Sigma-Aldrich; taurine (2-aminoethanesulfonic acid), methionine, DTNB [5,5'-dithiobis(2-nitrobensoic acid)] and NaN_3 were from Fluka; stock 30% H_2O_2 was from Merck.

Hypochlorite concentration was determined by OCl^- absorption at pH 12, assuming $\varepsilon_{290} = 350 \, M^{-1} \, cm^{-1}$ [24]. The TNB solutions were prepared by adjusting DTNB to pH 12 for 5 min and neutralizing to pH 7.4 [25], stored at 4°C and used within 3 days.

Proteins. Published procedures were used to obtain human MPO [16], Cp (in native state and upon limited proteolysis) [26], and Lf (>94% apo form) [27].

Chemiluminescence was assayed at room temperature using a SmartLum 5773 instrument (Interoptika-S, Russia). A standard sample contained 0.2 nM MPO and 10 μM luminol in 10 mM Na-P buffer pH 7.4 with 150 mM NaCl; additions or omissions are specified in the table and figure legends. Under constant stirring, the reaction was started by injecting H_2O_2 to a concentration of 5 μM. The result was recorded as integral CL over the first 10 s of the reaction.

Effect of omissions and additions in the MPO/chloride/luminol system on H_2O_2-induced chemiluminescence

Control	100 ± 18
Without chloride	24.7 ± 5.3
Without MPO	8.6 ± 0.9
With:	
Taurine (1 mM)	8.0 ± 0.5
Methionine (1 mM)	4.5 ± 0.4
Azide (1 mM)	6.8 ± 0.2
Guaiacol (2 μM)	2.4 ± 0.1
Guaiacol (2 μM), no chloride	2.6 ± 0.4
Guaiacol (50 μM)	7.2 ± 0.6

Chlorinating activity was determined by a standard technique [25] at 4 nM MPO and $2 \times 50 \, \mu M \, H_2O_2$ in 50 mM Na-P pH 7.4, 140 mM NaCl, with 10 mM taurine as the HOCl trap, finally adding TNB and measuring the loss of absorbance at 412 nm. The MPO activity thus calculated was 70 ± 10 mmol HOCl per mg protein in 10 min.

Peroxidase activity was measured with 0.5 mM ABTS or guaiacol and 25 nM MPO in 50 mM Na-P pH 7.4. After adding 0.1 mM H_2O_2, absorbance at respectively 405 and 450 nm was recorded with a Beckman DU-65 spectrophotometer. MPO activity with ABTS was 340 μmol/(min mg protein). The assay with tyrosine was run under the same conditions; the fluorescence of the oxidation product was excited at 325 nm and recorded at 410 nm with a Hitachi F4000 spectrofluorimeter.

Statistical assessment. All data are given as means of three measurements with standard deviation. With the Student's t-test, all differences mentioned were significant at $p < 0.05$.

RESULTS AND DISCUSSION

Chemiluminescence Assay for MPO Activity

A typical kinetic curve for CL evoked by H_2O_2 in the MPO/chloride/luminol system is shown in Fig. 2a. The flash is intense and short, most of the light being emitted in the first 2–3 s. As the shape of the curve did not change in our experiments (e.g. Fig. 2), all CL data are further given as the integral glow over 10 s.

The analysis performed to elucidate the nature of this CL is summarized in the table. Without the enzyme, the flash area was less than one-tenth; without chloride, it was only one-fourth. The reaction was drastically suppressed by MPO inhibitor azide and by hypochlorite scavengers taurine or methionine. These results indicate that the observed CL is largely due to MPO performing the halogenation cycle, i.e. com-
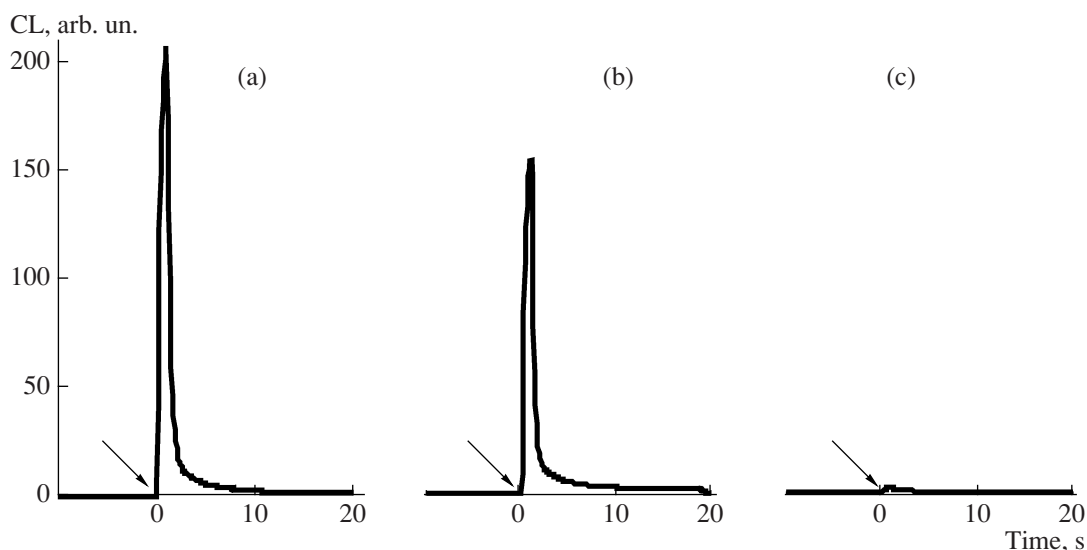
CL, arb. un.

Fig. 2. Typical kinetic curves of chemiluminescence evoked by adding H_2O_2 (arrow) into the MPO/chloride/luminol system: (a) control, (b) plus ceruloplasmin (0.1 nM), or (c) plus taurine (1 mM).

pound I oxidizing chloride to HOCl, which then oxidizes luminol.

This conclusion is supported by the effect of guaiacol, a substrate of the peroxidase cycle. We have found that, even at a physiological level of chloride, guaiacol almost completely switches MPO into the peroxidase mode [28]. The table shows that as little as 2 µM guaiacol suppressed the CL by 98%, regardless of the presence of chloride. Raising guaiacol to 50 µM did not appreciably raise the CL, confirming that in this assay the glow is unrelated to the peroxidase function.

Guaiacol can itself react with HOCl [29], but scavenging would be negligible, because the reaction rate constant for such phenol derivatives is 40–50 M^{-1} s^{-1} [30] while for luminol it is $5 \cdot 10^5$ M^{-1} s^{-1} [31]. It only

Fig. 3. Integral CL (recorded as in the MPO assay) versus the concentration of HOCl added to 10 µM luminol in 10 mM Na-P buffer pH 7.4.

remains to conclude that MPO prefers to oxidize guaiacol whereby, in accordance with Fig. 1, compound I turns into compound II (the respective redox potential is 1.35 V [32]) while the halogenation cycle is idled.

Figure 3 shows the CL dependence on the concentration of HOCl added to a solution of luminol; the plot is linear in the range of non-excessive concentrations (inset). Hence, the integral CL recorded in our MPO assay can serve as a measure of its halogenation activity.

MPO Activity in the Presence of Cp and/or Lf

To assess the influence of Cp, it was added to MPO in various amounts under conditions favoring complex formation [16]. [It was first ascertained that in all MPO assays the enzymic activity of Cp itself was negligible.]

The CL assay revealed that already at a Cp/MPO molar ratio of 0.5 the activity declined by 25–30% (a typical response is shown in Fig. 2b), and less than half remained at Cp/MPO ~ 3 (Fig. 4a, curve *1*). Under the same conditions, equivalent amounts of Cp subjected to limited proteolysis and containing no intact 132-kDa molecules had no appreciable effect (curve *2*). The conventional assay of MPO chlorinating activity with taurine gave the same patterns but at far higher molar ratios (Fig. 4b).

Previous studies have shown that intact Cp also suppresses the MPO peroxidase activity with ABTS, while cleaved Cp is ineffective, though both make complexes with MPO [33]. In all probability, limited proteolysis impairs a Cp region that is not decisive for MPO binding but is essential for inhibition. To add, ABTS oxidation by horseradish peroxidase was not affected by intact Cp (results not shown), so it is unlikely that the Cp copper ions could somehow interfere with the
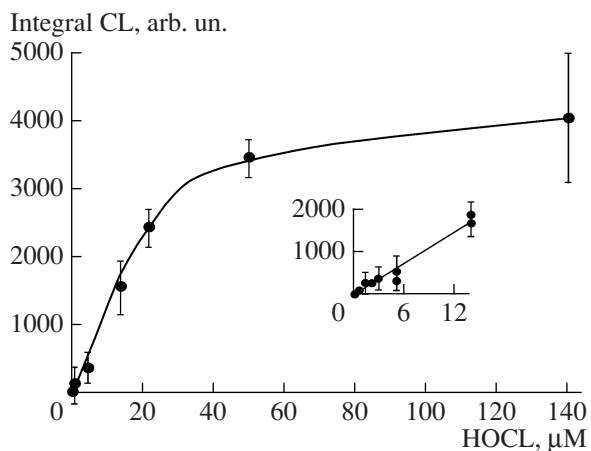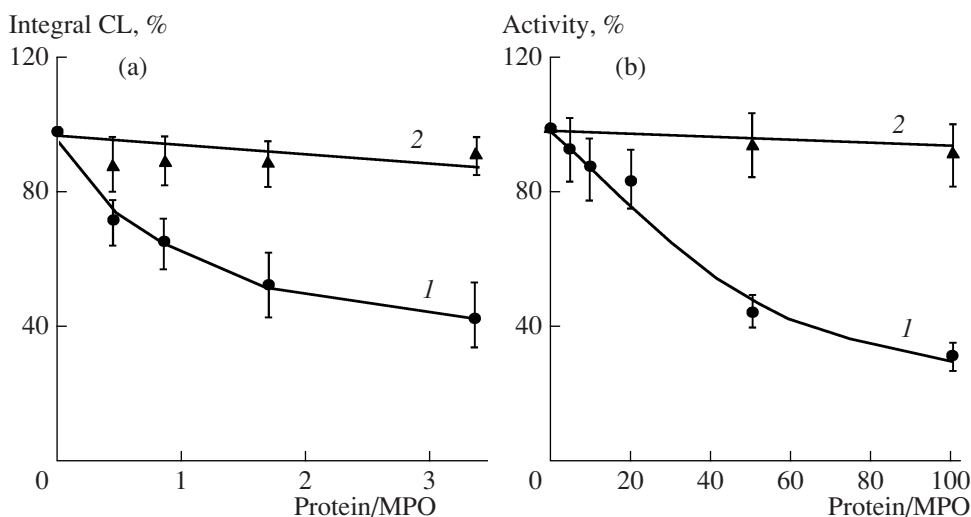
Fig. 4. Dependence of (a) integral CL and (b) taurine-chlorinating activity of MPO (relative to control) on the molar ratio of (*1*) native or (*2*) partly proteolyzed Cp to MPO.
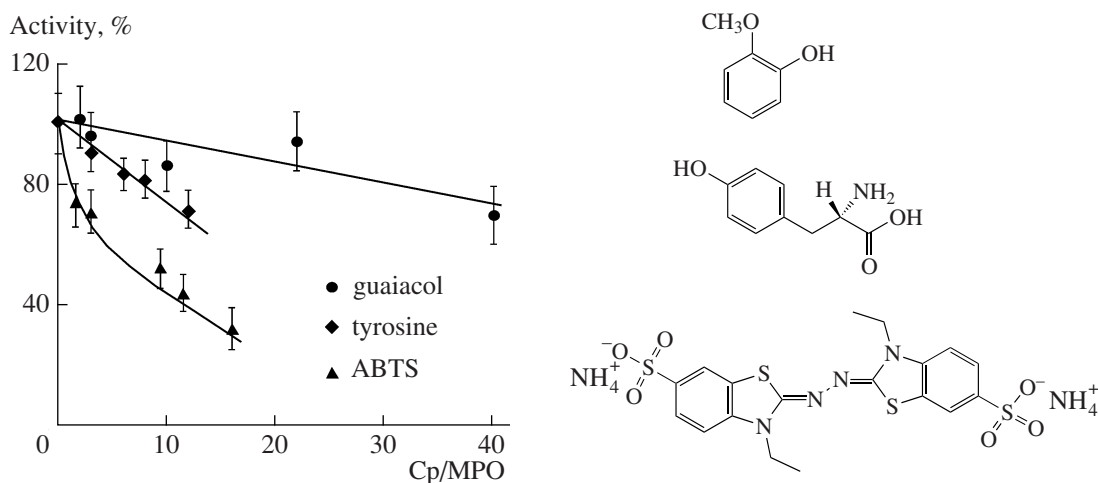

Fig. 5. Dependence of the peroxidase activity of MPO (relative to controls) on the Cp/MPO molar ratio for substrates of different size.

enzyme function. Again, suppression of the CL or chlorinating activity by Cp can hardly be attributed to simple interception of HOCl, because in this respect the cleaved Cp should not have differed much from the intact protein.

Thus, the most plausible idea is that MPO–Cp complexing creates steric hindrances to substrate binding. This is corroborated by the comparative influence of Cp on MPO peroxidase activity with substrates of different size (Fig. 5): there is but little effect on oxidation of small guaiacol; with slightly larger tyrosine the decrease is already reliable; and with large ABTS the effect is much more pronounced, as regards both the extent of inhibition and the Cp/MPO ratio at which it is observed.

The other tested protein, Lf, is a moderately basic protein and does not directly bind with MPO, but they can unite in a triple complex with Cp [16]. As summarized in Fig. 6, (apo)Lf neither itself influenced the MPO activity nor interfered with MPO blocking by Cp in three different assays, regardless of the Cp/MPO ratio or the Lf concentration (roughly equimolar to MPO).

Comparison of MPO Assay Efficacy

We used three different tests for MPO activity, of which the new CL assay and the conventional taurine assay pertain to the same halogenation function. A most important issue from this study is that the inhibitory effect of Cp in both conventional assays is observed only at a considerable molar excess (Figs. 4b, 5, 6b, c; see also [11, 17, 18]), whereas CL registers it at stoichiometric ratios (Figs. 2b, 4a or 6a) compatible with the naturally occurring MPO–Cp and MPO–Cp–Lf com-
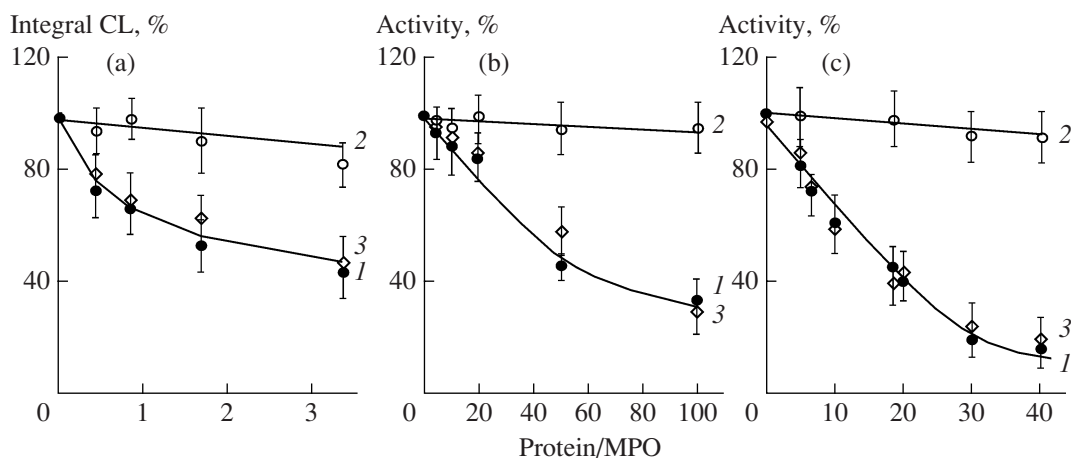
Fig. 6. Dependence of (a) integral CL, (b) taurine-chlorinating activity, and (c) ABTS-peroxidase activity of MPO (relative to controls) on the molar ratio (*1*) Cp/MPO, (*2*) Lf/MPO, or (*3*) Cp/MPO in the presence of (a) 0.34 nM, (b) 4 nM, and (c) 25 nM Lf.

plexes. Note that the CL assay allows measuring the MPO chlorination activity at a concentration well below the sensitivity threshold of the usual taurine assay. This makes it a convenient tool for examining the MPO behavior and influence of various modulators, as demonstrated here for ceruloplasmin, which obstructs the access of substrates to the MPO active site.

REFERENCES

1. S. J. Klebanoff, J. Leukoc. Biol. **77**, 598 (2005).
2. V. Vassiliev, Z. L. Harris, and P. Zatta, Brain Research Reviews **49**, 633 (2005).
3. P. F. Levay and M. Viljoen, Haematologica **80**, 252 (1995).
4. A. J. Kettle and C. C. Winterbourn, Redox Report **3**, 3 (1997).
5. P. G. Furtmüller, U. Burner, and C. Obinger, Biochemistry **37**, 17923 (1998).
6. O. M. Panasenko, J. Arnhold, and V. I. Sergienko, Biol. Membrany **19**, 403 (2002).
7. D. I. Pattison and M. J. Davies, Curr. Med. Chem. **13**, 3271 (2006).
8. D. I. Pattison and M. J. Davies, Biochemistry **43**, 4799 (2004).
9. O. Skaff, D. I. Pattison, and M. J. Davies, Chem. Res. Toxicol. **20**, 1980 (2007).
10. O. M. Panasenko, T. Vakhrusheva, V. Tretyakov, et al., Chem. Phys. Lipids **149**, 40 (2007).
11. W. Y. S. Park, K. Suzuki, S. Mumby, et al., Free Rad. Res. **33**, 261 (2000).
12. V. B. Vasil'ev, A. M. Kachurin, and N. V. Soroka, Biokhimiya **53**, 2051 (1988).
13. J. M. C. Gutteridge, R. Richmond, and B. Halliwell, FEBS Lett. **112**, 269 (1980).
14. Y. S. Park, K. Suzuki, N. Taniguchi, and J. M. C. Gutteridge, FEBS Lett. **458**, 133 (1999).
15. C. K. Mukhopadhyay, E. Ehrenwald, and P. L. Fox, J. Biol. Chem. **271**, 14773 (1996).
16. A. V. Sokolov, M. O. Pulina, K. V. Ageeva et al., Biokhimiya **72**, 506 (2007).
17. M. Segelmark, B. Persson, T. Hellmark, and J. Wieslander, Clin. Exp. Immunol. **108**, 167 (1997).
18. S. V. Griffin, P. T. Chapman, E. A. Lianos, and C. M. Lockwood, Kidney Intern. **55**, 917 (1999).
19. B. N. Borregaard and J. B. Cowland, Blood **89**, 3503 (1997).
20. D. Legrand, A. Pierce, E. Elass, et al., Adv. Exp. Med. Biol. **606**, 163 (2008).
21. N. Yu. Govorova, S. N. Lyzlova, B. P. Sharonov, and O. Yu. Yankovskii, Biokhimiya **52**, 1670 (1987).
22. O. M. Panasenko, J. Arnhold, Yu. A. Vladinirov, et al., Biofizika **40**, 1234 (1995).
23. D. I. Roshchupkin, N. S. Belakina, and M. A. Murina, Biofizika **51**, 99 (2006).
24. J. C. Morris, J. Phys. Chem. **70**, 3798 (1966).
25. A. J. Kettle and C. C. Winterbourn, Meth. Enzymol. **233**, 502 (1994).
26. A. V. Sokolov, M. O. Pulina, E. T. Zakharova, et al., Biokhimiya **70**, 1231 (2005).
27. E. T. Zakharova, M. M. Shavlovski, M. G. Bass, et al., Arch. Biochem. Biophys. **374**, 222 (2000).
28. I. I. Vlasova, J. Arnhold, A. N. Osipov, and O. M. Panasenko, Biokhimiya **71**, 825 (2006).
29. J. Michalowicz, W. Duda, and J. Stufka-Olczyk, Chemosphere **66**, 657 (2007).
30. D. I. Pattison and M. J. Davies, Chem. Res. Toxicol. **14**, 1453 (2001).
31. T. E. Eriksen, J. Lind, and G. Merenyi, J. Chem. Soc. Faraday Trans. **77**, 2125 (1981).
32. P. G. Furtmüller, J. Arnhold, W. Jantschko, et al., Biochem. Biophys. Res. Comm. **301**, 551 (2003).
33. A. V. Sokolov, Candidate's Dissertation in Biology (St. Petersburg, Inst. Exp. Med., 2007).