

## Kinetic Investigation of Myeloperoxidase upon Interaction with Copper, Cadmium, and Lead Ions

Maryam Shabani, Mohsen Ani, Ahmad Movahedian and Seyed Ziyae Aldin Samsam Shariat\*

Dept. of Clinical Biochemistry, Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Received 24 April 2011; revised 20 August 2011; accepted 3 September 2011

### ABSTRACT

**Background:** Myeloperoxidase (MPO), which is abundantly expressed in neutrophils, catalyzes the formation of a number of reactive oxidant species. However, evidence has emerged that MPO-derived oxidants contribute to tissue damage and initiation and propagation of inflammatory diseases, particularly, cardiovascular diseases. Therefore, studying the regulatory mechanisms of the enzyme activity is of great importance. For clarifying some possible mechanism of the enzyme activity, kinetic investigations of MPO in the presence of Copper (Cu), Cadmium (Cd), and Lead (Pb) ions were carried out *in vitro*. **Methods:** MPO was partially purified from human white blood cells using ion-exchange and gel-filtration chromatography techniques. Its activity was measured spectrophotometrically by using tetramethyl benzidine (TMB) as substrate. **Results:** Purified enzyme had a specific activity of 21.7 U/mg protein with a purity index of about 0.71. Cu inhibited MPO activity progressively up to a concentration of 60 mM at which about 80% of inhibition achieved. The inhibition was non-competitive with respect to TMB. An inhibitory constant ( $K_i$ ) of about 19 mM was calculated from the slope of repot. Cd and Pb did not show any significant inhibitory effect on the enzyme activity. **Conclusion:** The results of the present study may indicate that there are some places on the enzyme and enzyme-substrate complex for Cu ions. Binding of Cu ions to these places result in conformational changes of the enzyme and thus, enzyme inhibition. This inhibitory effect of Cu on the enzyme activity might be considered as a regulatory mechanism on MPO activity. *Iran. Biomed. J.* 15 (3): 107-112, 2011

**Keywords:** Myeloperoxidase, Copper (Cu), Enzyme inhibition, Cadmium (Cd), Lead (Pb)

### INTRODUCTION

Myeloperoxidase (MPO) is the most abundant protein of the azurophilic granules of neutrophils [1] that is found in macrophages [2], microglia [3], Kupffer cells [4], and neurons [5]. MPO is capable of catalyzing the oxidation of chloride and bromide ions by  $H_2O_2$  generating hypochlorous acid (HOCl) and hypobromous acid (HOBr), respectively [6, 7]. At physiological conditions, HOCl, which is known as a potent oxidant, is the predominant product of the enzyme [8]. The resultant hypohalogenite (HOCl and HOBr) react readily with a wide variety of biomolecules such as proteins [9], lipids [10], DNA [11], and lipoproteins [12, 13]. In addition, increased systemic MPO activity has been associated with the production of various tissue injury and diseases, including atherosclerosis [14], vasculitis [15], stroke [16], cancer [17], Parkinson's disease [18],

Alzheimer's disease [5] and multiple sclerosis [19]. Therefore, it is plausible to suggest that increased activity of MPO may cause the induction of the disease, and thus the regulation of the enzyme activity is useful in prevention of the disease. It is reported that the activity of MPO is affected by many biologically active agents [20]. Among heavy metals, copper (Cu) is of particular concern as an essential trace element and Cadmium (Cd) and lead (Pb) are two environmentally persistent toxic elements which are non-essential in human. Cu ions act as cofactors in many enzymes, such as Cu/Zn superoxide dismutase, cytochrome C oxidase, amino acid oxidase, and laccase [21]. At the cellular level, Cu also plays an essential role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron metabolism [21]. In addition, some enzymes are inhibited by Cu ions. For example, glutamate dehydrogenase and

\*Corresponding Author; Tel. (+98-311) 7922589; Fax: (+98-311) 6680011; E-mail: samsam@pharm.mui.ac.ir

urease activity are inhibited by Cu ions. Chen *et al.* [23] also showed that digestive enzymes activities in the guts of various marine invertebrates are inhibited in the presence of Cu. Although many physiological functions of Cu have already been clarified, scientists are still uncovering the new information regarding the functions of Cu in the human body.

Cd and Pb, two persistent and common environmental contaminants, affect cell metabolism and catalytic activity of several enzymes through binding to thiol groups on protein molecules [24, 25]. Pandya *et al.* [25] have studied the effect of Pb and Cd on the activity of steroid metabolizing enzymes, 17- $\beta$  hydroxyl steroid oxidoreductase and uridine diphosphate glucuronyltransferase, and showed the inhibitory effects of the metals ions on the enzyme activities. However, few studies have been carried out on the effect of heavy metals on MPO activity.

Due to important role of MPO in inducing various diseases, regulatory mechanisms of the enzyme activity have attracted much attention. In this regard and for better understanding of the possible mechanisms by which these metals affect on the enzyme activity, changes in the kinetic parameters of MPO upon interaction with Cu, Cd, and Pb ions was studied.

## MATERIALS AND METHODS

**Materials.** Human blood was obtained from Isfahan Blood Transfusion Organization (Iran), tetramethyl benzidine (TMB),  $H_2O_2$ , ammonium sulfate, Cu sulfate ( $CuSO_4$ ), Cd chloride ( $CdCl_2$ ), Pb acetate  $Pb(CH_3COO)_2$  cetyltrimethylammonium bromide (CTAB), and Sephadex G150 were obtained from Sigma Chemical Co. (USA). All other chemicals were reagent grade.

### Methods:

**Buffy coat isolation.** MPO was purified from normal human leukocytes using a method described by Morita *et al.* [26]. Buffy coat, the principal source of white blood cells, was separated by centrifugation of citrated blood at  $1000 \times g$  for 15 min. The layer between plasma and red cells (buffy coat) was collected and kept for analysis.

**Myeloperoxidase purification.** After lysis of the buffy coat by adding 0.5% CTAB, MPO was isolated and cell debris was removed by centrifugation at  $15,000 \times g$  at  $5^\circ C$  for 15 min. All subsequent centrifugation was carried out under these conditions. The supernatant was treated with solid ammonium sulfate to yield a final concentration of 50% saturation. This solution was kept at  $4^\circ C$  for 30 min and then centrifuged to remove the precipitate. The resulting

supernatant was treated with solid  $(NH_4)_2SO_4$  to increase the concentration to 65% saturation, and incubated at  $4^\circ C$  for 30 min prior to centrifugation. Precipitated MPO was re-dissolved in a buffer containing 50 mM Tris pH 7.0 and dialyzed against the same buffer. The dialysate was applied to the first CM-Sephadex column ( $4 \times 50$  cm) equilibrated with the dialysis buffer to remove the surfactant from the enzyme solution. The column was washed until the absorption at 280 nm returned to the baseline and then the enzyme was eluted with 0.3 M dipotassium hydrogen phosphate solution. The enzyme fractions were then applied to the second CM-Sephadex column ( $4 \times 50$  cm) with a linear gradient of 0.05 M to 0.3 M dipotassium hydrogen phosphate solution. The MPO fractions were then subjected to gel filtration on a column ( $2.5 \times 100$  cm) of Sephacryl S-300.

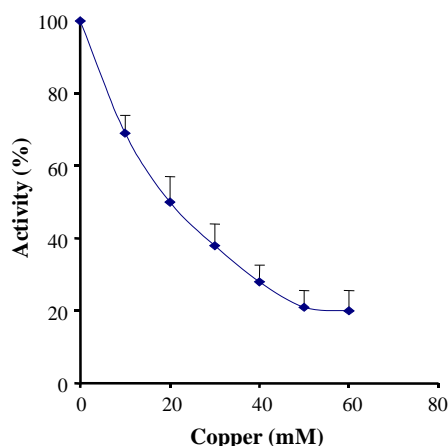
**Enzyme assay.** MPO activity was measured by the method reported by Suzuki *et al.* [27]. Then, 0.88 mM TMB, 1 mM of  $H_2O_2$  and 50  $\mu l$  of enzyme solution were added to 1 ml of reaction mixture containing 50 mM acetate buffer to initiate the reaction. The absorbance was recorded at 655 nm during 3 min using a Perkin-Elmer 515 UV/VIS spectrophotometer. Protein concentration was determined by the Lowry [28] procedure with bovine serum albumin as standard.

**Kinetic studies.** Kinetic investigations of human leukocyte-derived MPO was performed by adding different concentrations of the metal ions to the assay mixture and incubating at  $25^\circ C$  for 10 min. Lineweaver-Burk plot and the corresponding replot were drawn using linear regression analysis.

**Statistical analysis.** Student's *t*-test was used for the statistical analysis. Data are expressed as mean  $\pm$  SD.  $P < 0.05$  was considered statistically significant.

## RESULTS

Results of this investigation showed that MPO from whole human blood was purified with a purity index of about 0.71 and had a specific activity of 21.7 U/mg protein. It was also shown that Cu inhibited human leukocytes MPO activity progressively up to 60 mM where 80% inhibition was achieved 10 min after addition of Cu ions (Fig. 1). Lineweaver-Burk reciprocal plot of MPO in the presence of two fixed concentrations of Cu is shown in Figure 2. It can be seen that the mode of inhibition is non-competitive, meaning that Cu could probably bind either to the free form of the enzyme or to the enzyme-substrate complex, producing binary and ternary complexes respectively. An inhibitory constant ( $K_i$ ) of 19 mM



**Fig. 1.** Concentration-dependent inactivation of myeloperoxidase by Cu. Copper ions were added to the assay mixture and after 10-minute incubation at room temperature, the enzyme activity was measured. Values represent mean  $\pm$  SD of three independent experiments.

was calculated from the slope replot (Fig. 3). When MPO was incubated in the presence of different concentrations of Cd or Pb, no significant changes were seen between MPO activity in the absence or presence of these metals ions (Fig. 4).

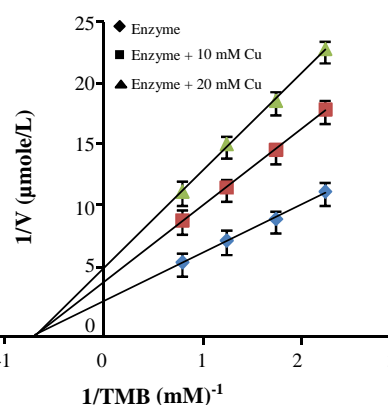
## DISCUSSION

The mechanisms underlying MPO oxidizing activity is a complex process thus, kinetic investigations about its behavior in the presence of various ligands may help to clarify this mechanism. One of the most common groups of ligands which are used in kinetic investigations of enzymes is heavy metals. Among these metals Cu, Pb, and Cd are concerns of many investigators due to their widespread distributions. Cu, is known to be an integral component of several enzymes, and an essential trace element in human, while Cd and Pb seem to be non-essential elements and highly toxic for general metabolism.

The present study was carried out on human MPO in the presence of Cu, Cd, and Pb to clarify kinetic. It was shown that interaction of Cu with human MPO led to a reversible inhibition of the enzyme activity. Cu inhibited the enzyme activity in a dose-dependent manner up to 60 mM concentrations where about 80% inhibition achieved. Lineweaver-Burk plot of the kinetic data showed that the mode of inhibition in the presence of Cu is non-competitive. In the other hand, Cu binds to both the enzyme and enzyme-substrate complex, produces conformational changes close to the enzyme active site of MPO and impedes the binding of substrates to the active center of the MPO, and therefore enzyme inactivation. Different mechanisms

have been reported for the influences of Cu on various enzymes activity. For example, Mengyao *et al.* [29] have reported that S-adenosylhomocysteine hydrolase activity is inhibited in the presence of Cu ions and the mode of inhibition was non-competitive. They showed that binding of Cu ions to this enzyme resulted in the release of  $\text{NAD}^+$  cofactors, explaining the less of the enzyme activity [29]. Tormanen [30] has studied inhibition of rat liver and kidney arginase by Cu ions and has shown that the kinetics of the inhibition was non-linear allosteric.

Lisa [31] has studied directly the kinetic and equilibrium effects of  $\text{Cu}^{2+}$  on the enzyme activity and showed that at equilibrium, binding of two to three  $\text{Cu}^{2+}$  stoichiometrically to the enzyme resulted in enzyme inactivation. Hadzi-Taskovic sukulovic *et al.* [32] has shown that peroxidative and oxidative catalytic functions of class III peroxidase were inhibited by Cu. They showed that this inhibition was reversible, and accompanied by disappearance of some and appearance of new isoforms of the enzyme. Sokolov *et al.* [33] have shown that ceruloplasmin, a Cu containing metalloenzyme which is synthesized in liver and carries approximately 95% of total plasma Cu, inhibits MPO activity. Kinetic studies have shown that ceruloplasmin behaves as a competitive inhibitor of MPO [33]. In the other hand, ceruloplasmin binds to the active site of the MPO and impedes the binding of substrates to the active center of MPO, and therefore enzyme inactivation. It is very interesting that ceruloplasmin and MPO in complex affect the enzymatic activity of each other [33]. Although Cu ions inhibited MPO activity, Cd and Pb could not do so. There are reports indicating that Cd and Pb ions can

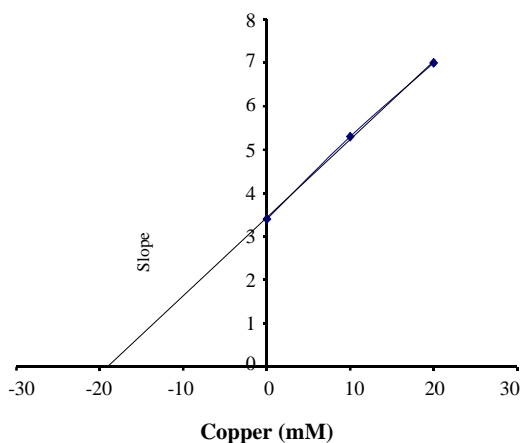


**Fig. 2.** Lineweaver-Burk reciprocal plot of the myeloperoxidase activity versus the reciprocal concentration of tetramethyl benzidine in the absence ( $\blacklozenge$ ), or 10 mM ( $\blacksquare$ ) and 20 mM ( $\blacktriangle$ ) concentrations of copper, as an inhibitor. Each point represents the average of three measurements. behavior of MPO *in vitro*.

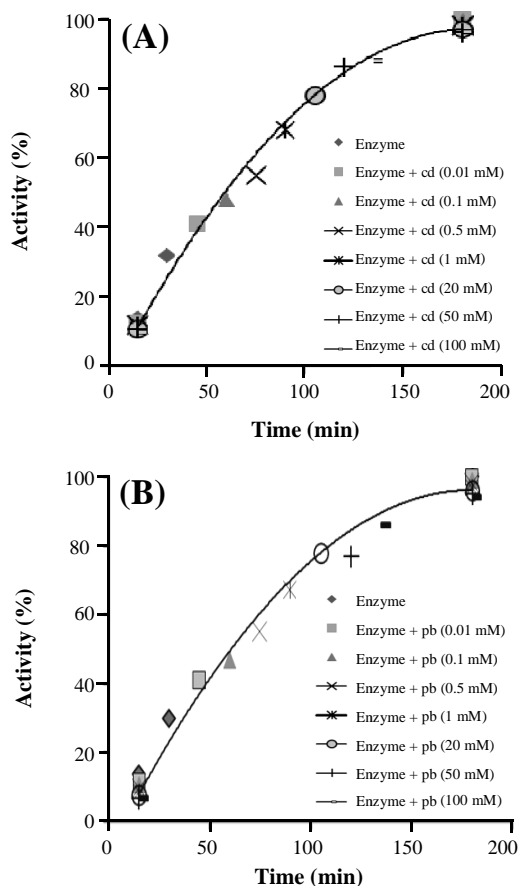
bind to enzymes having functional thiol groups, and effectively inactivate them. Thus, the presence of thiol groups in MPO which are able to bind to Cd or Pb seems to be far from the active site of the enzyme and this explains why the activity of this enzyme is not very much affected. Therefore, conformational changes upon Cd and Pb binding to the enzyme are not able to change active site of the enzyme that impedes the substrate binding to the active center of enzyme.

As mentioned before, MPO is abundantly expressed in neutrophils and, to a lesser extent, in monocytes and certain type of macrophages. Elevated serum activity of MPO has been reported in many inflammatory diseases, particularly cardiovascular and neurological diseases [34]. Therefore, therapies capable of suppressing MPO activity may be useful in controlling diseases associated with elevated MPO levels. Although many metals are involved in changes in activity of many enzymes, little is understood about their effect on MPO activity. As a result, it is plausible to investigate the MPO kinetic in the presence of the metal ions to clarify the mechanisms by which these affects are brought about.

According to the results presented in our study, there may be some places both on the free enzyme molecules and enzyme-substrate complex for Cu ions. Binding of the metal ions to these places may induce conformational changes in the enzyme molecule, leading to reversible enzyme inhibition. Due to the inhibition of the enzyme in the presence of Cu ions and also inhibition of the enzyme in the presence of ceruloplasmin, a ferroxidase that contains greater than 95% of plasma Cu, it is suggested that Cu may be a biological regulator of activated neutrophils. Moreover, it may be proposed that Cu ions bound to the ceruloplasmin molecules may be the cause of the inhibitory effect of ceruloplasmin on MPO activity.



**Fig. 3.** Replot of inverse slope of the Lineweaver- Burk plot vs. Cu concentration. Values were obtained from Figure 2.



**Fig. 4.** The effect of different cadmium (A) and lead (B) concentrations on myeloperoxidase activity. Cadmium chloride and lead acetate were added to the assay mixture and incubated at 25°C for 10 min. The enzyme activity was measured as described in Methods at intervals of 15 min for 180 min.

## ACKNOWLEDGMENTS

This work was supported by the Research Council of Isfahan University of Medical Sciences (research no. 388302).

## REFERENCES

1. Klebanoff, S.J. (2005) Myeloperoxidase: friend and foe. *J. Leukoc. Biol.* 77: 598-625.
2. Sugiyama, S., Okada, Y., Sukhova, G.K., Virmani, R., Heinecke, J.W. and Libby, P. (2001) Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am. J. Pathol.* 158: 879-891.
3. Gray, E., Thomas, T.L., Betmouni, S., Scolding, N. and Love, S. (2008) Elevated activity and microglial expression of myeloperoxidase in demyelinated cerebral

- cortex in multiple sclerosis. *Brain Pathol.* 18: 86-95.
4. Brown, K.E., Brunt, E.M. and Heinecke, J.W. (2001) Immunohistochemical detection of myeloperoxidase and its oxidation products in kupffer cells of human liver. *Am. J. Pathol.* 159: 2081-2088.
  5. Green, P.S., Mendez, A.J., Jacob, J.S., Crowley, J.R., Growdon, W., Hyman, B.T. and Heinecke, J.W. (2004) Neuronal expression of myeloperoxidase is increased in Alzheimer's disease. *J. Neurochem.* 90: 724-733.
  6. Senhilmohan, R. and Kettle, A.J. (2006) Bromination and chlorination reaction of myeloperoxidase at physiological concentrations of bromide and chloride. *Arch. Biochem. Biophys.* 445: 235-244.
  7. Panasenkov, O.M., Vakhrusheva, T., Tretyakov, V., Spalteholz, H. and Arnhold, J. (2007) Influence of chloride on modification of unsaturated phosphatidylcholines by the myeloperoxidase hydrogen peroxide/bromide system. *Chem. Phys. Lipids* 149: 40-51.
  8. Winterbourn, C.C. and Kettle, A.J. (2000) Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic. Biol. Med.* 29: 403-409.
  9. Pattison, D.I., Hawis, C.L. and Davies, M.J. (2007) Hypochlorous acid-mediated protein oxidation: How important are chloramines transfer reactions and protein tertiary structure. *Biochemistry* 46: 9853-9864.
  10. Spickett, C.M., Jerlich, A., Panasenkov, O.M., Arnold, J. Pitt, A.R., Stelmazynska, T. and Schaur, R.J. (2000) The reactions of hypochlorous acid, the reactive oxygen species produced by myeloperoxidase, with lipids. *Acta Biochim. Pol.* 47: 889-899.
  11. Suzuki, T. (2006) DNA damage and mutation caused by vital biomolecules, water, Nitric oxide, and hypochlorous acid. *Genes Environ.* 28: 48-55.
  12. Marsche, G., Furtmuller, P.G., Obinger, C., Sattler, W., and Malle, E. (2008) Hypochlorite-modified high-density lipoprotein acts as a sink for myeloperoxidase *in vitro*. *Cardiovasc. Res.* 79: 187-194.
  13. Bergt, C., Pennathur, S., Fu, X., Byun, J., O'Brien, K., McDonald, T.O., Singh, P., Anantharamaiah, G.M., Chait, A., Brunzell, J., Geary, R.L., Oram, J.F. and Heinecke, J.W. (2004) The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci USA.* 101: 13032-13037.
  14. Zhang, R., Brennan, M.L., Fu, X., Aviles, R.J., Pearce, G.L., Penn, M.S., Topol, E.J., Sprecher, D.L. and Hazen, S.L. (2001) Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* 286: 2136-2142.
  15. Ishida-Okawara, A., Oharaseki, T., Takahashi, K., Hashimoto, Y., Aratani, Y., Koyama, H., Maeda, N., Naoe, S. and Suzuki, K. (2001) Contribution of myeloperoxidase to coronary artery vasculitis associated with MPO-ANCA production. *Inflammation* 25: 381-387.
  16. Re, G., Azzimondi, G., Lanzarini, C., Bassein, L., Vaona, I. and Guarnierimieri, C. (1997) Plasma lipoperoxidative markers in ischaemic stroke suggest brain embolism. *Eur. J. Emerg. Med.* 4: 5-9.
  17. Trush, A., Esterline, R.L., Mallet, W.G., Mosebrook, D.R. and Twerdok, L.E. (1991) Further evidence for the role of myeloperoxidase in the activation of benzo [a] pyrene-7, 8-dihydrodiol by polymorphonuclear leukocytes. *Adv. Exp. Med. Biol.* 283: 399-401.
  18. Choi, D.K., Pennathur, S., Perier, C., Tieu, K., Teismann, P., Wu, D.C., Jackson-Lewis, V., Vila, M., Vonsattel, J.P., Heinecke, J.W. and Przedborski, S. (2005) Ablation of the inflammatory enzyme myeloperoxidase mitigates features of parkinson's disease in mice. *J. Neurosci.* 25: 6594-6600.
  19. Hoy, A., Leininger-Muller, B., Kutter, D., Siest, G. and Visvikis, S. (2002) Growing significance of myeloperoxidase in non-infectious disease. *Clin. Chem. Lab Med.* 40: 2-8.
  20. Choi, D.K., Koppula, S., Choi, M. and Suk, K. (2010) Recent developments in the inhibitors of neuroinflammation and neurodegeneration: inflammatory oxidative enzyme as a drug target. *Expert Opin. Ther. Pat.* 20: 1531-1546.
  21. Yruela, I. (2005) Copper in plants. *Braz. J. Plant Physiol.* 17: 145-156.
  22. Faixova, Z. and Faix, S. (2002) Influence of metal ions on ruminal enzyme activities. *Acta Vet. Brno.* 71: 451-455.
  23. Chen, Z., Mayer, L.M., Weston, D.P., Bock, M.J. and Jumars, P.A. (2002) Inhibition of digestive enzyme activities by copper in the guts of various marine benthic invertebrates. *Environ. Toxicol. Chem.* 21: 1243-1248.
  24. Ahamed, M., Verma, S., Kumar, A. and Siddiqui, M.K. (2005) Environmental exposure to lead and its correlation with biochemical indices in children. *Sci Total Environ.* 346: 48-55.
  25. Pandya, C.D., Pillai, P.P. and Gupta, S.S. (2010) Lead and cadmium co-exposure mediated toxic insults on hepatic-steroid metabolism and antioxidant system of adult male rats. *Biol. Trace Elem. Res.* 134: 307-317.
  26. Morita, Y., Iwamoto, H., Aibara, S., Kobayashi, T. and Hasegawa, E. (1986) Crystallization and properties of myeloperoxidase from normal human leukocytes. *J. Biochem.* 99: 761-770.
  27. Suzuki, K., Ota, H., Sasagawa, S., Sakatani, T. and Fujikura, T. (1983) Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal. Biochem.* 132: 345-352.
  28. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1957) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
  29. Li, M., Li, Y., Chen, J., Wei, W., Pan, X., Liu, J., Liu, Q., Leu, W., Zhang, L., Yang, X., Lu, J. and Wang, K. (2007) Copper ions inhibit S-adenosylhomocysteine hydrolase by causing dissociation of NAD<sup>+</sup> cofactor. *Biochemistry* 46: 11451-11458.
  30. Tormanen, C.D. (2001) Allosteric inhibition of rat liver and kidney arginase by copper and mercury ions. *J. Enzyme Inhib.* 16: 443-449.
  31. Stone, L.A., Jackson, G.S., Collinge, J., Wadsworth, J.D. and Clarke, A.R. (2007) Inhibition of proteinase K activity by copper (II) ions. *Biochemistry* 46: 245-252.
  32. Hadzi-Taskovic Sukalovic, V., Vuletic, M., Veljovic-Jovanovic, S. and Vucinic, Z. (2010) The effects of manganese and copper *in vitro* and *in vivo* on

- peroxidase catalytic cycles. *J. Plant Physiol.* 167: 1550-1557.
33. Sokolov, A.V., Ageeva, K.V., Pulina, M.O., Cherkalina, O.S., Samygina, V.R., Vlasova, I.I., Panasenko, O.M., Zakharova, E.T. and Vasilyev, V.B. (2008) Ceruloplasmin and myeloperoxidase in complex affect the enzymatic properties of each other. *Free Radic Res.* 42 (11-12): 989-998.
34. Rodriguez, E., Nilges, M., Weissleder, R. and Chem, J.W. (2010) Activatable magnetic resonance imaging agents for myeloperoxidase sensing: mechanism of activation, stability, and toxicity. *J. Am. Chem. Soc.* 132:168-177.