Asian Journal of Chemistry Vol. 19, No. 7 (2007), 5587-5595

Two Different Behaviours of Mushroom Tyrosinase on the Impact of Different Concentrations of Thiophenol in Acidic Medium

S. ZOLGHADRI and A.A. SABOURY*
Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
Fax: (98)(21)66404680; Tel: (98)(21)66956984; E-mail: saboury@ut.ac.ir.

The effect of thiophenol on the kinetic of coumaric acid hydroxylation by mushroom tyrosinase has been investigated at 20°C in 10 mM phosphate buffer solution, pH 5.3. The results show that thiophenol can activate or inhibit the cresolase activity of mushroom tyrosinase depending to the concentration of thiophenol. It was proposed that the enzyme has two distinct sites for thiophenol. The first one is a high-affinity activation site and the other is a low-affinity inhibition site. Activation of the enzyme in the low concentration of thiophenol arises from increasing the affinity of binding for the substrate as well as increasing the enzyme catalytic constant. The resulting fluorescence spectra at low concentrations of the ligand clearly demonstrate typical enhancement of aromatic emission in the presence of thiophenol referring to the more exposed residues after the addition of thiophenol. Although the tertiary structure of the enzyme changes due to the activation of mushroom tyrosinase by binding of thiophenol, but it is not accompanied by a change in the secondary structure of mushroom tyrosinase.

Key Words: Mushroom tyrosinase, Thiophenol, Benzenethiol, Competitive inhibition, Activation, Fluorescence, Circular dichroism.

INTRODUCTION

Tyrosinase or polyphenoloxidase (EC 1.14.18.1) is a copper-containing enzyme, responsible for the formation of the pigments of skin, hair, and eye[1-6]. This enzyme uses molecular oxygen to catalyze two different reactions *i.e.*, the oxidation of monophenols, *e.g.*, tyrosine, to their corresponding *o*-diphenols (monophenolase or cresolase activity) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity). *o*-Quinones are highly reactive substances, which polymerize spontaneously to macromolecules like melanin[7].

Although the physiological function of tyrosinase in fungi is not yet understood, melanin synthesis is correlated with the differentiation of reproductive organs and spore formation, the virulence of pathogenic fungi, and tissue protection after injury[8-13].

Common mushroom tyrosinase (MT) from the species *Agaricus bisporus* with a molecular mass of 120 kD, is composed of two H subunits (43 kD) and two L subunits (13 kD) and contains two active sites[13,14]. Its active site has a di-copper center, resembling that of hemocyanins[15], but not identical[16]. Each copper ion in the active site is coordinated by three nitrogen atoms coming from three adjacent histidine residues and the enzyme can experience three forms of met, oxy and deoxy[17,18].

In some vegetables and fruits, tyrosinase is responsible for browning and is considered to be deleterious to the colour quality of plant-derived foods and beverages. This unfavourable darkening from enzymatic oxidation generally results in loss of nutritional and market value. Therefore, the browning is a major problem in the food industry and the control of the tyrosinase activity is important in preventing the synthesis of melanin in the browning of vegetables and fruits[14,19]. The unfavourable browning caused by tyrosinase on the surface of seafood products has also been of great concern[20]. In addition, tyrosinase inhibitors have become increasingly important in medicinal[21] and cosmetic[22] products in relation to hyperpigmentation.

To understand the mechanism of enzyme action and inhibition, an attempt has been made to obtain additional information about the structure, function and relationship of MT[23-27]. After introducing two new bi-pyridine synthetic compounds as potent uncompetitive MT inhibitors[28], the inhibitory effects of three synthetic *n*-alkyl dithiocarbamates, with different tails, were elucidated[29]. Recently, we used thiophenol to clarify the binding process for cresolase and catecholase inhibition of the enzyme is different[30]. In the present investigation, it is found that the enzyme inhibition and activation by thiophenol can be occurred in relative high (90-400 nM) and low (0-50 nM) concentration, respectively.

EXPERIMENTAL

Mushroom tyrosinase (MT; EC 1.14.18.1), specific activity 3400 units/mg, was purchased from Sigma. Coumaric acid was taken from the authentic samples. Analytical grade of thiophenol was prepared from Sigma. Phosphate buffer (10 mM, pH 5.3) was used throughout this research and the corresponding salts were obtained from Merck. All experiments were carried out at 20ºC.

Kinetic measurements: Kinetic assay of cresolase activity was carried out through depletion of coumaric acid, for 15 min, enzyme concentration of 78.24 µg/mL, at 288 nm wavelengths using a Cary spectrophotometer, 100 Bio-model, with jacketed cell holders. Freshly prepared enzyme, substrate, thiophenol were used in this work. All the enzymatic reactions were run in phosphate buffer (10 mM) at pH 5.3 in a conventional quartz

cell thermostated to maintain the temperature at 20 ± 0.1°C. Substrate addition was followed after incubation of enzyme with different concentrations of thiophenol.

Circular dichroism spectroscopy: The far-UV CD region (190-260), which corresponds to peptide bond absorption, was analyzed by an Aviv model 215 spectropolarimeter (Lakewood, NJ, USA) to give the content of regularly secondary structure in MT. Protein solutions were prepared in the buffer. The protein solutions of 0.2 mg/mL without and with incubation at different concentrations of thiophenol (20, 60, 330 and 1000 nM) for at least 4 min were used to obtain the spectra. All spectra were collected in a triplicate from 190 to 260 nm and a back ground-corrected against buffer blank. The results were expressed as ellipticity (cm^2 $dmol^{-1}$) based on a mean amino acid residue weight (MRW) of 125 for MT having the average molecular weight of 120 kDa[31]. The molar ellipticity was determined as $[\theta] = (100 \times (MRW) \times \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/mL and l is the length of the light path in cm.

Intrinsic fluorescence: Intrinsic fluorescence intensity measurements were carried out using a Hitachi spectrofluorimeter, MPF-4 model, equipped with a thermostatically controlled cuvette compartment. The intrinsic emission of protein, 0.17 mg/mL, was seen at the excitation wavelength of 280 nm. The experiments were repeated in the presence of different concentrations of thiophenol (20, 60, 330 and 1000 nM).

RESULTS AND DISCUSSION

The effect of thiophenol on the cresolase activity was examined at pH 5.3 and 20°C at two ranges of relatively high (90-400 nM) and low (0-50 nM) concentrations of thiophenol. Double reciprocal Lineweaver-Burk plots for the cresolase activity of MT on hydroxylation of *p*-coumaric acid, as the substrate, in the presence of different concentrations of thiophenol at high concentrations have been shown in Fig. 1. The Figure shows a series of straight lines intersects each other exactly on the vertical axis. The maximum velocity (V_{max}) has not been changed by thiophenol but the apparent Michaelis constant (K'_m) value has been increased, which confirms the competitive mode of inhibition. The insect on Fig. 1 shows the secondary plot, the slope (K'_m/V_{max}) at any concentration of the inhibitor *vs.* concentration of inhibitor, which gives the inhibition constants (K_i) from the abscissa-intercepts. The K_i value is 0.08 μM. Hence, the association binding constant, as the inverse of K_i, equals to 12.5 μM^{-1}.

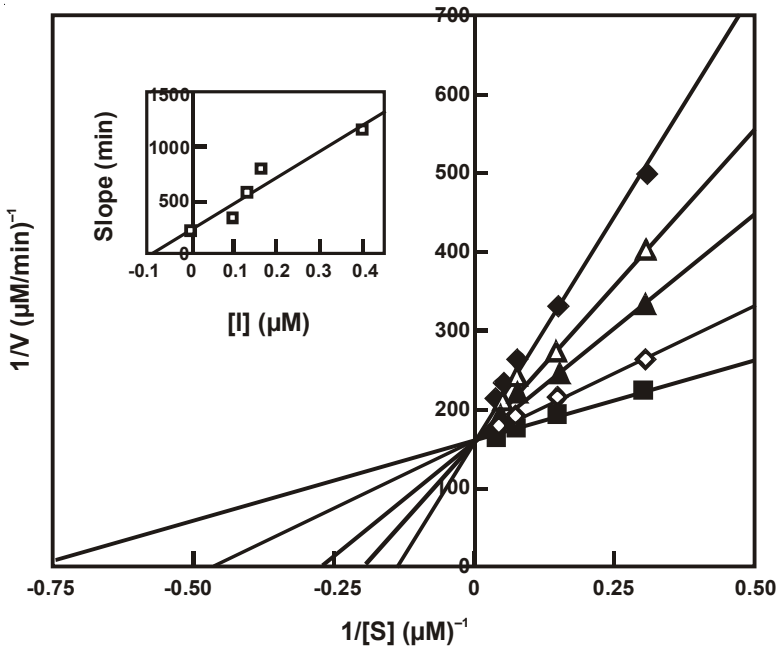


Fig. 1. Double reciprocal Lineweaver-Burk plots of MT kinetic assays for cresolase reactions of *p*-coumaric acid in 10 mM phosphate buffer, pH = 5.3, at 20°C and 78.24 μM enzyme concentration, in the presence of different fixed concentrations of thiophenol: 0 nM (■), 99 nM (◇), 132 nM (▲), 162 nM (△), 400 nM (◆). The insect on Fig. 1 shows the secondary plot, the slope (K'_m/V_{max}) at any concentration of the inhibitor *vs.* concentration of inhibitor (I)

Fig. 2 shows the double reciprocal Lineweaver-Burk plots for the cresolase activity of MT on hydroxylation of *p*-coumaric acid in the presence of different relatively low concentrations of thiophenol. The figure shows a series of straight lines intersects each other on the left hand side of the vertical axis, over the horizontal axis, indicating activation of MT at low concentrations of thiophenol. The kinetic pathway of activation follows the general non-essential activation system. The apparent maximum velocity (V_{max}') and apparent dissociation constant of the substrate, S, (K_S') values can be obtained at any fixed concentration of thiophenol from the vertical (Y) and abscissa (X) intercept, respectively. The rapid-equilibrium model has been proposed to describe the non-essential activation of an enzyme by an activator molecule A (**Scheme-I**)[32]. In this model,

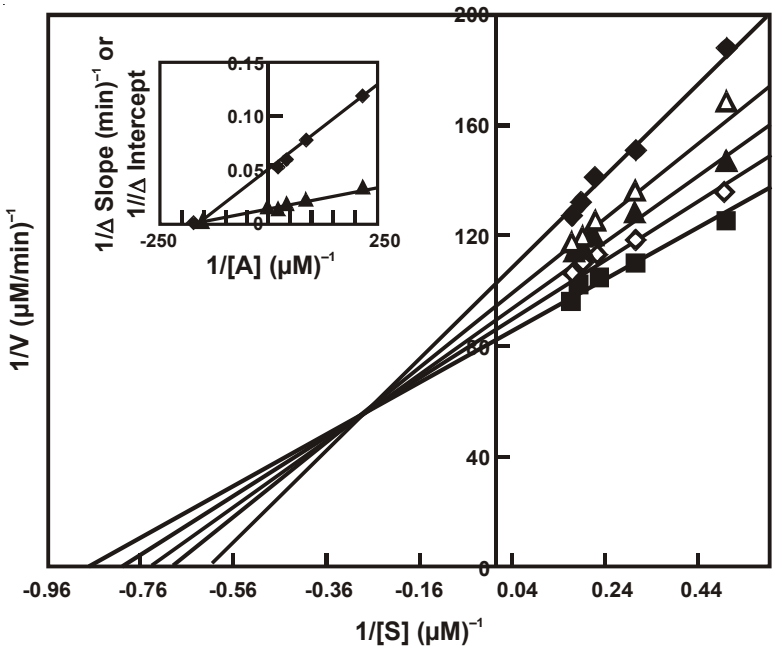
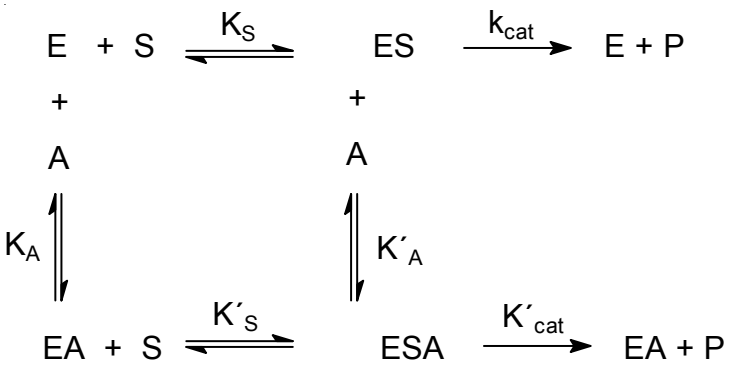


Fig. 2. MT kinetic assays for cresolase reactions of *p*-coumaric acid in 10 mM phosphate buffer, pH = 5.3, at 20°C and 78.24 μM enzyme concentration, in the presence of different fixed concentrations of thiophenol: 45 nM (■), 23 nM (◇), 12 nM (▲), 5 nM (△), 0 nM (◆). The insect on Fig. 2 shows the secondary plot, 1/ΔY-intercept against 1/ΔA (◆) and 1/Δ slope against 1/ΔA (▲)



Scheme-I

$V_{max}' = V_{max}(1 + \beta[A]/\alpha K_A)/(1 + [A]/\alpha K_A)$ and $K_S' = K_S(1+ [A]/\alpha K_A)/(1 + [A]/\alpha K_A)$. K_S is the dissociation constant of the substrate from the enzyme, K_A is the dissociation constant of the effector from the enzyme, α and β represent maximal changes in K_S and V_{max} of the enzyme in the presence of thiophenol, respectively and [A] is the concentration of thiophenol as the activator. V_{max} and K_S values are obtained from the Y-intercept and X-intercept of the Lineweaver-Burk linear plot, respectively, in the absence of the activator. Then, the slope and Y-intercept changes in the Lineweaver-Burk plot due to the presence of thiophenol at different concentration are obtained and replotted their inverse *vs.* inverse concentration of the activator as the secondary plot to find α, β and K_A values. The linear plot of $1/\Delta$ slope against $1/\Delta A$ shows the Y-intercept of $\beta V_{max}/(\beta-1)$ and the X-intercept of $-\beta/\alpha K_A$. The linear plot of $1/\Delta$Y-intercept against $1/\Delta A$ shows the Y-intercept of $\beta V_{max}/K_S(\beta-\alpha)$ and the X-intercept of $-\beta/\alpha K_A$, α and β values were found to be 0.7 and 1.25, respectively and K_S and K_A were found to be 1.64 and 0.01 μM, respectively. The α value ($\alpha < 1$) obtained herein suggests that the binding of thiophenol to the enzyme can increase the binding affinity of the substrate. The β value ($\beta > 1$) obtained also suggests that the binding of thiophenol to the enzyme can increase the maximum velocity of the enzyme due to the increase of the enzyme catalytic constant (k_{cat}).

The results show that thiophenol can activate and inhibit the enzyme in a concentration dependent manner. The affinity for binding of thiophenol as an activator is greater than the affinity for binding of thiophenol as an inhibitor ($K_A < K_S$). MT should include two distinct sites for thoiphenol, the first one is for activation and the second is for inhibition. The first one may be close enough to the binuclear centre to interact allosterically with the substrate. The inhibition should be related to a competition with the substrate for the active site because it appears at high thiophenol concentrations. The activation-inhibition found should be the result of the competition between these two different binding sites.

Similar evidence was obtained from the fluorescence spectroscopic studies. Fig. 3 supports observable changes in the tertiary structure of MT in the presence of thiophenol at pH = 5.3 in the high and low concentrations of thiophenol. At low concentrations (≤ 0.3 μM), thiophenol binding to MT increased the intensity of fluorescence emission. Further increase in thiophenol concentration (up to *ca.* 1 μM) produced binding of thiophenol accompanied by decrease in the fluorescence emission. It is proposed that thiophenol interacts with two classes of binding sites which activate and inhibit the MT, respectively. It is also worthy to note that CD spectra rules out the possible changes in the secondary structure of MT in the presence of thiophenol at pH = 5.3 (Fig. 4).

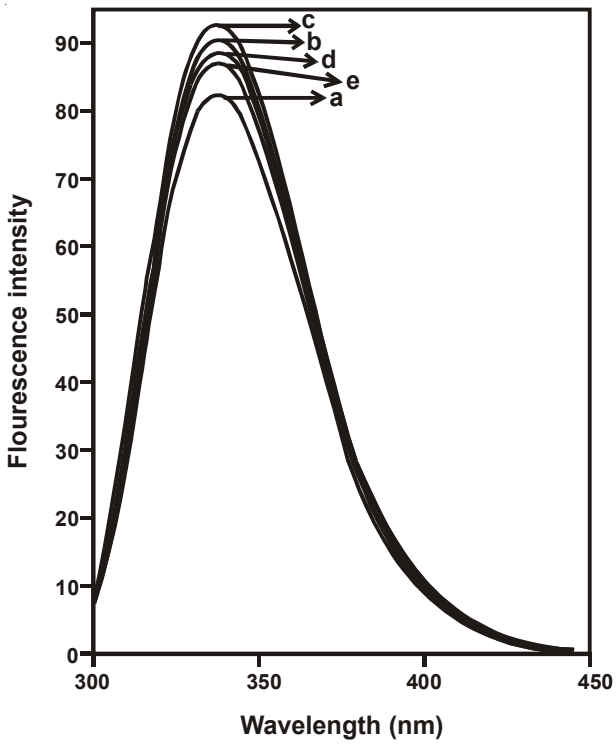


Fig. 3. Intrinsic fluorescence emission spectra of MT (a) and MT in the presence of different concentration of thiophenol: 20 nM (b), 60 nM (c), 330 nM (d) and 1000 nM (e) at pH = 5.3. The excitation wavelength was 280 nm. The concentration of the enzyme is 0.17 mg/mL

As a conclusion, thiophenol can bind to the two different separate binding sites of the enzyme. The affinity of binding for the first binding site ($1/K_A$ = 100 μ M^{-1}) is eight times greater than the second binding site ($1/K_i$ = 12.5 μ M^{-1}) so that the occupation of the first binding site is occurred at the first, which the enzyme becomes more active at low concentration of thiophenol. The occupation of the second site is occurred at high concentration of thiophenol, which leads to the inhibition of MT. The activation site of the enzyme by thiophenol may be close enough to the binuclear centre to interact allosterically with the substrate binding site. Occupation of the activation site of MT by thiophenol is accompanied by a change in the tertiary structure of the enzyme without any change in the second structure. Binding of thiophenol as an activator causes not only increasing the affinity of binding for the substrate by 1.4 (α = 0.7; $1/\alpha$ = 1.4) but also increasing the catalytic constant of MT (k_{cat}) by 1.25

(β = 1.25). The inhibition of MT should be related to a competition of thiophenol with the substrate for the binding to the active site of the enzyme. The affinity of binding for the substrate ($1/K_S$ = 0.61 μ M^{-1}) is lower than the affinity of binding of thiophenol either in the activation or in the inhibition site.

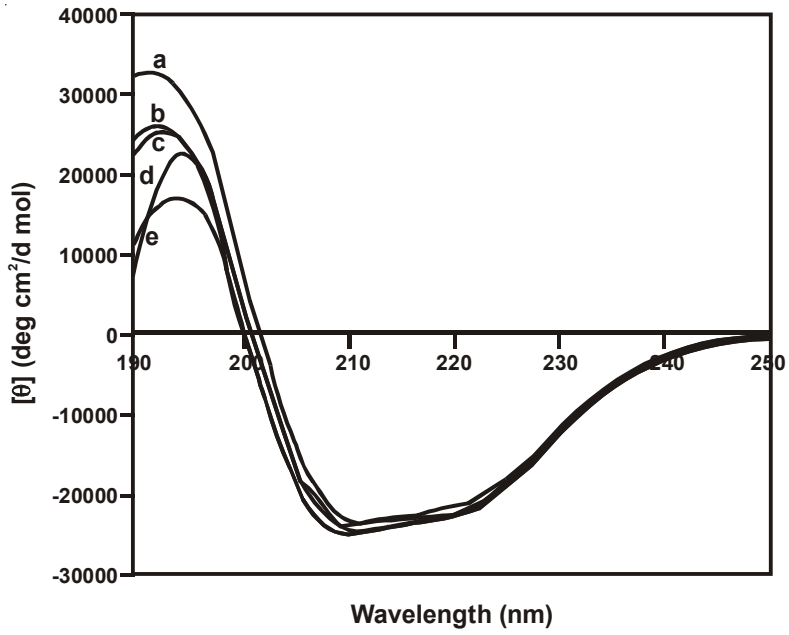


Fig. 4. UV-CD spectra registered for MT (a) and MT in the presence of different concentration of thiophenol: 20 nM (b) 60 nM (c) 330 nM (d) and 1000 nM (e) at pH = 5.3

ACKNOWLEDGEMENTS

The financial support given by the University of Tehran and the Iran National Science Foundation (INSF) are gratefully acknowledged.

REFERENCES

1. A. Sanchez-Ferrer, J.N. Rodriguez-Lopez, F. Garcia-Canovas and F. Garcia-Carmona, *Biochim. Biophys. Acta*, **1247**, 1 (1995).
2. C.W.G. Van Gelder, W.H. Flurkey and H.J. Wichers, *Phytochemistry*, **45**, 1309 (1997).
3. A.J. Winder and H. Harris, *Eur. J. Biochem.*, **198**, 317 (1991).
4. D.A. Robb, Copper Proteins and Copper Enzymes, Boca Raton, CRC Press (1984).
5. P.A. Riley, *J. Theor. Biol.*, **203**, 1 (2000).
6. L.G. Fenoll, J.N. Rodriguez-Lopez, F. Garcia-Sevilla, J. Tudela, P.A. Garcia-Ruiz and R. Varon, *Eur. J. Biochem.*, **267**, 5865 (2000).

7. E.J. Land, C.A. Ramsden and P.A. Riley, *Acc. Chem. Res.*, **36**, 300 (2003).
8. G.M. Gadd, *FEMS Microbiol. Lett.*, **9**, 237 (1980).
9. L.A. Cooper and G.M. Gadd, *J. Microbiol. Serol.*, **50**, 53 (1984).
10. A.A. Bell and M.H. Wheeler, *Annu. Rev. Phytopathol.*, **24**, 411 (1986).
11. R.J. Howard, M.A. Ferrari, D.H. Roach and N.P. Money, *Proc. Natl. Acad. Sci. (U.S.A.)*, **88**, 11281 (1991).
12. W.C. Zimmerman, R.A. Blanchette, T.A. Burnes and R.L. Farrel, *Mycologia*, **87**, 857 (1995).
13. M. Miranda, O. Zarivi, A. Bonfigli, F. Amicarelli, P. Aimola, A.M. Ragnelli and G. Pacioni, *Pigm. Cell Res.*, **10**, 46 (1997).
14. M.V. Martynez and J.R. Whitaker, *Trends Food Sci. Technol.*, **6**, 195 (1995).
15. S.J. Orlow, Z. Bao-Kang, A.K. Chakraborty, M. Dricker, S. Pifko-Hirst and J.M. Pawelek, *J. Investig. Dermatol.*, **103**, 196 (1994).
16. C. Eicken, B. Krebs and J.C. Sacchettini, *Curr. Opin. Struct. Biol.*, **9**, 677 (1999).
17. C.J. Cooksey, P.J. Garratt, E.J. Land, C.A. Ramsden and P.A. Riley, *Biochem. J.*, **333**, 685 (1998).
18. A. Rescigno, F. Sollai, B. Pisu, A. Rinaldi and E. Sanjust, *J. Enz. Inhib. Med. Chem.*, **17**, 207 (2002).
19. M. Friedman, *J. Agric. Food Chem.*, **44**, 631 (1996).
20. M. Ogawa, N.B. Perdigao, M.E. Santiago and T.T. Kozima, *Bull. Japan Soc. Sci. Fish.*, **50**, 1763 (1984).
21. K. Maeda and M. Fukuda, *J. Soc. Cosmet. Chem.*, **42**, 361 (1991).
22. A.M. Mayer and E. Harel, in ed.: P.F. Fox, Phenoloxidases and Their Significance in Fruit and Vegetables, Food Enzymology, Elsevier, London, UK, pp. 373-398 (1998).
23. K. Haghbeen, A.A. Saboury and F. Karbassi, *Biochim. Biophys. Acta*, **1675**, 139 (2004).
24. F. Karbassi, K. Haghbeen, A.A. Saboury, B. Ranjbar and A.A. Moosavi-Movahedi, *Coll. Surf. B: Biointerfaces*, **32**, 137 (2003).
25. S.S. Borojerdi, K. Haghbeen, A.A. Karkhane, M. Fazli and A.A. Saboury, *Biochem. Biophys. Res. Commun.*, **314**, 925 (2004).
26. N. Gheibi, A.A. Saboury, K. Haghbeen and A.A. Moosavi-Movahedi, *Coll. Surf. B: Biointerfaces*, **45**, 104 (2005).
27. F. Karbassi, K. Haghbeen, A.A. Saboury, B. Ranjbar, A.A. Moosavi-Movahedi and B. Farzami, *Int. J. Biol. Macromol.*, **34**, 257 (2004).
28. F. Karbassi, A.A. Saboury, M.T.H. Khan, M.I. Choudhary and Z.S. Saifi, *J. Enz. Inhib. Med. Chem.*, **19**, 349 (2004).
29. N. Gheibi, A.A. Saboury, H. Mansury-Torshizy, K. Haghbeen, A.A. Moosavi-Movahedi, *J. Enz. Inhib. Med. Chem.*, **20**, 393 (2005).
30. A.A. Saboury, S. Zolghadri, K. Haghbeen and A.A. Moosavi-Movahedi, *J. Enz. Inhib. Med. Chem.*, **21**, 711 (2006).
31. K.J. Strothkemp, R.L. Jolley and H.S. Mason, *Biochim. Biophys. Res. Commun.*, **70**, 519 (1976).
32. V. Leskovac, Comprehensive Enzyme Kinetics, Kluwer Academic/Plenum Publisher, New York, Ch. 7, p. 111 (2003).

(*Received*: 18 December 2006; *Accepted*: 19 June 2007) AJC-5742