ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 1, pp. 97-101. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 1, pp. 124-130.

Catalytic Activity and Stability of Xanthine Oxidase in Aqueous–Organic Mixtures

M. R. Rashidi[1]*, M. H. Soruraddin[2], F. Taherzadeh[2], and A. Jouyban[3]

[1]*Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz 51664-14766, Iran; E-mail: rashidi@tbzmed.ac.ir*
[2]*Analytical Chemistry Department, School of Chemistry, Tabriz University, Tabriz 51664-14766, Iran*
[3]*Pharmaceutical Chemistry Department, School of Pharmacy, Tabriz University of Medical Sciences, Tabriz 51664-14766, Iran*

Received December 11, 2007
Revision received July 28, 2008

Abstract—In the present study, bovine milk xanthine oxidase activity in various aqueous–organic mixtures and the effects of pH, temperature, and lyophilization on the enzyme activity have been investigated. The enzyme was incubated with xanthine as the substrate in Sorenson's phosphate buffer (pH 7.0) containing 0.1 mM EDTA, and the activity was determined spectrophotometrically in the absence and presence of different fractions of nine water-miscible organic solvents at 27-50°C and at different pH values ranging from 6 to 9. The organic solvents reduced the enzyme activity to different extents. In spite of these inhibitory effects, the enzyme showed relatively good stability in the aqueous–organic mixtures compared with the aqueous medium. A significant increase in the activity of the lyophilized enzyme was observed in pure organic solvents.

DOI: 10.1134/S0006297909010155

Key words: xanthine oxidase, aqueous–organic mixtures, enzyme activity, thermal stability, lyophilization

A growing interest in developing aqueous–organic and non-aqueous media for enzymatic reactions is seen in the literature, and the use of enzymes in these media has found a variety of practical applications. Recently, organic solvents have been used to increase the yields of enzymatic reactions [1, 2], alter enzyme specificity [3, 4], and enhance enzyme stability [5, 6]. Aqueous–organic and non-aqueous media have also been used to overcome some difficulties that can occur for enzyme catalytic reactions in aqueous solutions, such as insolubility of substrate, unfavorable thermodynamic equilibria, and difficult product recovery [7]. However, enzyme activity may be dramatically reduced in the presence of organic solvents. Therefore, a clear understanding of the interaction of organic solvents with enzymatic processes would be of great value and provide useful information for many practical purposes.

Xanthine oxidase (EC 1.17.3.2) is a molybdenum-containing enzyme that plays an important role in the metabolism of many xenobiotics and drugs, such as purines and pyrimidines [8, 9], 6-mercaptopurine and azathioprine [10], thiazides [11], pyrazinamide [12], and acyclovir [13]. This enzyme also serves as an important biological source of reactive oxygen species that are involved in many pathological processes, such as inflammation, atherosclerosis, cancer, and aging [14, 15]. Xanthine oxidase has been used in biosensors for estimation of fish meat freshness in the food industry [16], evaluation of ischemic myocyte injury [17], and measurement of theophylline [18].

In spite of these broad and important activities of xanthine oxidase, the activity of this enzyme in non-aqueous media has not received enough attention, and the study on the activity of xanthine oxidase in the presence of organic solvents is limited to few reports from the 1960s [19-21]. Furthermore, there were some limitations in these studies such as use of only lyophilized enzyme and measurement of the enzyme activity in anaerobic conditions using electron acceptors other than molecular oxygen [19-21]. However, the nature of the electron acceptor has a critical effect on the enzyme activity. Fridovich has indicated that dimethoxyethane is capable of increasing xanthine oxidase activity towards acetaldehyde in the presence of 2,6-dichloroindophenol [19].

Abbreviations: DMF, dimethylformamide; THF, tetrahydrofuran.

* To whom correspondence should be addressed.

When this electron acceptor was replaced with other electron acceptors including molecular oxygen, not only any enhancement of the rate by dimethoxyethane was eliminated, but also dimethoxyethane acted as an inhibitor. In the present study the activity of xanthine oxidase in different aqueous–organic solvents and the effects of some factors such as temperature, lyophilization, and pH on this activity are investigated.

MATERIALS AND METHODS

Chemicals. Xanthine and bovine milk xanthine oxidase (grade I: from buttermilk, 0.5 unit per mg protein) were obtained from Sigma-Aldrich (England). All other chemicals were purchased from Merck (Germany).

Enzyme assay. All spectrophotometric determinations were carried out using a Shimadzu 2550 UV/VIS (Japan) spectrophotometer, which was controlled by the Shimadzu UV Probe personal software package including kinetics software. The instrument was connected to a Shimadzu cell temperature control unit. Xanthine oxidase activity was determined spectrophotometrically at 295 nm through monitoring of uric acid production from xanthine as the substrate in the presence of molecular oxygen as the electron acceptor. Xanthine (20 μM) was incubated with the enzyme fraction in Sorenson's phosphate buffer (67 mM, pH 7.0) containing 0.1 mM EDTA, and the oxidation rates were measured up to 10 min.

Assay of xanthine oxidase activity in organic solvents. The activity of xanthine oxidase was also measured in different aqueous–organic media as described in the previous section. The solutions of organic solvents were prepared in 67 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA and their concentrations were varied from 0% to a concentration that gave almost complete inhibition of the enzyme activity. The solvents tested were acetonitrile, tetrahydrofuran (THF), 1-propanol, 2-propanol, ethanol, N,N-dimethylformamide (DMF), pyridine, dioxane, and methanol. The concentration of half-inactivation (C_{50}) for each organic solvent was determined by inspection of the plot of residual activity in aqueous–organic media against the percentage of the organic solvent [22].

Thermal stability of xanthine oxidase in aqueous–organic mixtures. A solution of the organic solvent in 67 mM Sorenson's phosphate buffer, pH 7.0, containing 0.1 mM EDTA was prepared by mixing a volume of the organic solvent that gives about 30% inhibition. This volume of each organic solvent was obtained from the assay of the enzyme activity in the presence of the solvents as described above. The final percentage of each organic solvent was as follows: acetonitrile (7.5%), THF (2.5%), 1-propanol (5%), 2-propanol (7.5%), ethanol (10%), DMF (7.5%), pyridine (2.5%), dioxane (7.5%), and methanol (2.5%). Then, 100 μl of enzyme solution (final concentration of 40 μg/ml) was separately incubated with each buffer–organic solvent mixture at 37°C for 24 h. Aliquots of the reaction solution were removed at various time intervals and added to a 20 μM solution of xanthine in the buffer and the change in absorbance was measured at 295 nm, reflecting the enzyme activity.

Effect of pH on xanthine oxidase activity in aqueous–organic mixtures. Because of the differences in the pH concept in aqueous and aqueous–organic mixtures, the apparent pH approach (pH*) was used to express the pH of the solvent mixtures [23]. The effect of pH* on xanthine oxidase activity was investigated by measuring the activity in 67 mM Sorenson's phosphate buffer and also in the aqueous–organic mixtures. Each organic solvent was mixed with the phosphate buffer to give 10, 20, and 30% of the mixture with the final pH* of the solution adjusted at 6, 7, 8, and 9.

Activity of lyophilized enzyme in organic solvents. First 100 μl of the enzyme solution containing 4 μg protein was lyophilized using a freeze dryer (Christ Alpha 1-4) in glass tubes. Then 3 ml of each organic solvent was separately added into the tube and incubated at 37°C for 5 h. The stock solution (6 mM) of xanthine was prepared in Sorenson's phosphate buffer pH 7.0. The reaction was started by the addition of 10 μl of the stock xanthine solution to the tube, and absorbance was followed at 295 nm for 10 min at 37°C. In these experiments the amount of water in the incubation medium would be 80 mg water per mg protein, or 0.18 M.

RESULTS

Xanthine oxidase activity in aqueous–organic mixtures. The effects of some water-miscible organic solvents on xanthine oxidase activity monitored at 295 nm are illustrated in Fig. 1. In all cases, the enzyme activity decreases with increasing organic solvent concentration. The largest decrease in the activity was observed for methanol ($C_{50} = 1.8 \pm 0.9\%$, v/v), while dioxane ($C_{50} = 15 \pm 3\%$) caused the smallest reduction. With methanol, pyridine ($C_{50} = 3 \pm 1\%$), THF ($C_{50} = 4 \pm 2\%$), and 1-propanol ($C_{50} = 7 \pm 3\%$), the reduction of the initial oxidation rate of xanthine occurred in a nonlinear manner; however, the reaction was inhibited almost linearly in the presence of the other solvents.

Xanthine oxidase activity at different temperatures in the presence of organic solvents. The enzyme activity steadily increased by 172% as temperature was elevated from 27 to 42°C in the absence of any organic solvent. The activity decreased at all temperatures as any of the organic solvents was included to the incubation solution. However, the effects of temperature on the enzyme activity in the aqueous–organic mixtures were complex.

As seen in Fig. 2, at 27°C the residual activity in all mixtures was higher than the corresponding values at

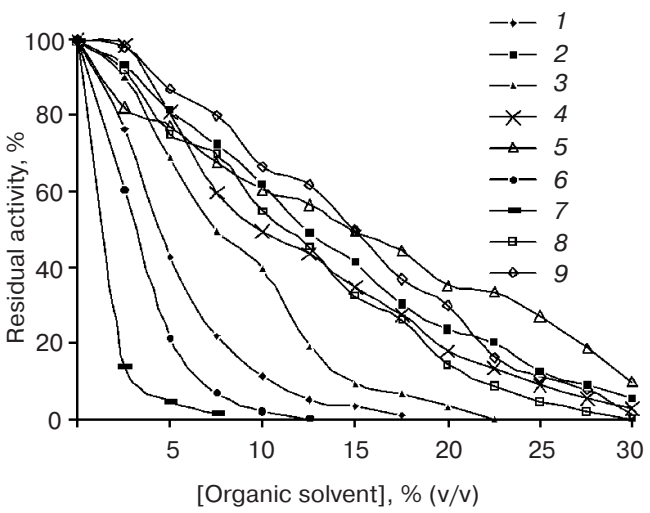

Fig. 1. Xanthine oxidase activity at 37°C and pH* 7 in the presence of different concentrations of organic solvents. Each point is the mean of triplicate assays where standard deviations were ± 5% of the experimental values. Here and in Figs. 2 and 4, solvents THF, DMF, 1-propanol, 2-propanol, dioxane, pyridine, methanol, acetonitrile, and ethanol are designated as *1-9*, respectively.

37°C. As the temperature was increased, the inhibitory effects of all organic solvents on the enzyme activity were increased. The relationship between temperature and the C_{50} was found to be linear for THF (r = –0.952), 1-propanol (r = –0.816), pyridine (r = –0.798), acetonitrile (r = –0.912), and ethanol (r = –0.968). However, for the remaining four solvents, the dependence of C_{50} on temperature was found to be low. When the analysis was carried out without the values at 50°C, the magnitude of the linearity was improved markedly for the all solvents except for methanol (r = –0.430 vs. –0.044) and acetonitrile (r = –0.912 vs. –0.889). This indicates that the behavior of xanthine oxidase in aqueous–organic media is relatively perturbed at higher temperatures and under this condition the ability of the organic solvent to interact with the enzyme through stripping water from the enzyme and/or competition with noncovalent interactions increases [24]. In Fig. 2, the graphical representation of the relationships between temperature and C_{50} values in nine aqueous–organic solvents is shown.

Thermal stability. Interestingly, incubation of xanthine oxidase in aqueous–organic mixtures not only did not result in a marked loss of the activity, but also with some organic solvents, such as dioxane, an increase in the activity was observed. A sharp decline occurred in the early time of the incubation with all organic solvents, but the activity immediately increased thereafter. In all cases, this elevation in the activity was linear (r > 0.950) and almost reached a plateau after 60-180 min (Fig. 3). The enzyme was quite stable even in the presence of methanol and pyridine, and more than 65% of the original activity was retained after a 24-h incubation with these two solvents, while the C_{50} values for these solvents were 2 ± 1 and 3 ± 1% (v/v), respectively. The highest (140%) activity was observed in dioxane (C_{50} = 15 ± 3%, v/v).

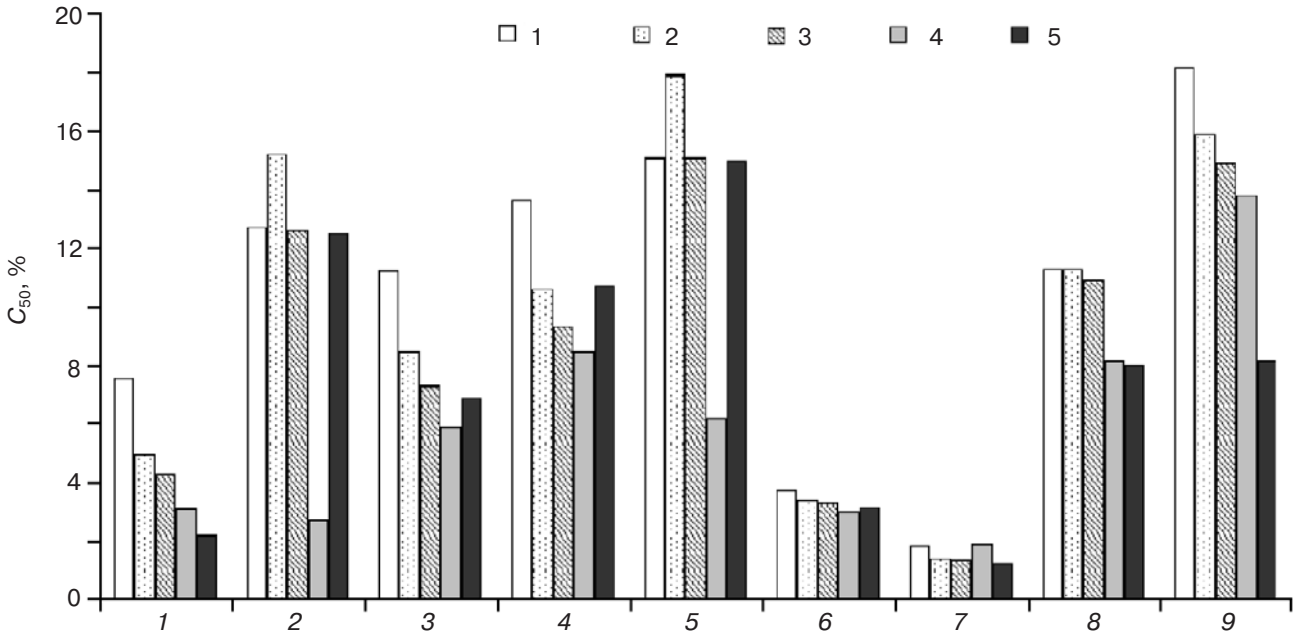

Fig. 2. Relationship between temperature and concentration of organic solvent causing 50% reduction in bovine milk xanthine oxidase activity in aqueous–organic mixtures (C_{50}). Enzyme concentration was 40 μg/ml. Temperatures: 1-5) 27, 32, 37, 42, and 50°C, respectively.

Effects of pH on xanthine oxidase activity in aqueous–organic mixtures. In the absence of the organic solvents, the xanthine oxidase activity increased 2.2-fold as the pH was increased from 6 to 8. Then, it decreased at pH 9 by 15%, giving the value of 8 as the optimum pH. However, the optimum pH for the xanthine oxidase activity was affected by the type of the organic solvent used in the reaction medium. Figure 4 illustrates the effect of pH* on xanthine oxidase activity in the presence of 10% organic solvent (v/v).

Activity of lyophilized xanthine oxidase in organic solvents. The activity of lyophilized xanthine oxidase was found to be higher in all the aqueous–organic solvents than in the phosphate buffer (Fig. 5). The highest activity was observed in DMF, followed by THF and ethanol. In these media, the activity increased by 7.1-, 3.4-, and 3.0-fold, respectively.

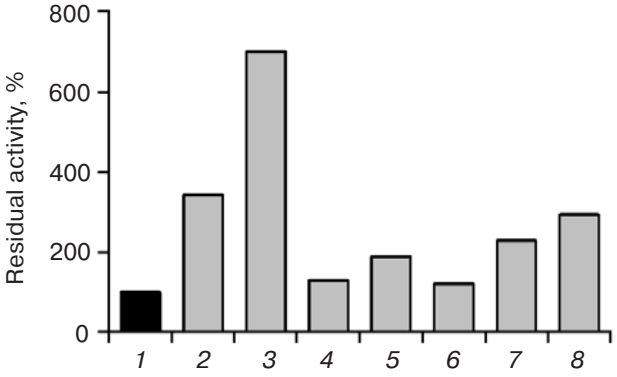

Fig. 5. Activity of lyophilized xanthine oxidase with 20 μM xanthine in different aqueous–organic solvents (pH* 7.0 at 37°C): *1*) buffer; *2*) THF; *3*) DMF; *4*) propanol; *5*) dioxane; *6*) methanol; *7*) acetonitrile; *8*) ethanol.

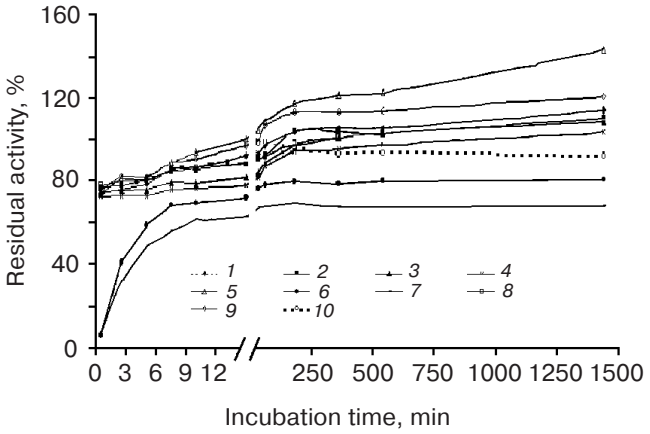

Fig. 3. Stability profile of bovine milk xanthine oxidase in aqueous–organic mixtures. The enzyme was incubated up to 24 h in various aqueous–organic mixtures at 37°C, and the activity was measured at different intervals during this period. *1-9*) Organic solvents (see designations in Fig. 1); *10*) phosphate buffer.

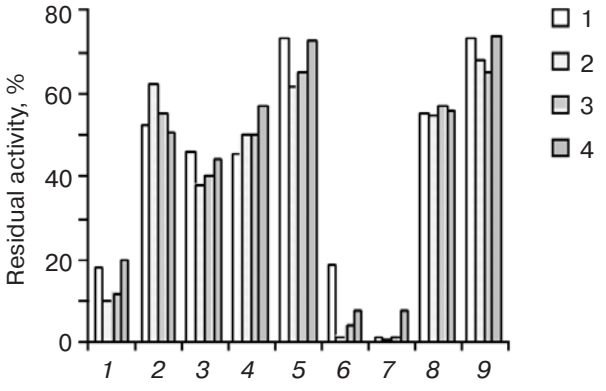

Fig. 4. Xanthine oxidase activity in aqueous buffer with 10% organic solvent (v/v) at different pH* values: 1) 6.0; 2) 7.0; 3) 8.0; 4) 9.0.

DISCUSSION

Xanthine oxidase is a molybdenum-containing cytosolic enzyme that can catalyze many important reactions [8-13, 16, 18]. In spite of increasing attempts to develop aqueous–organic and non-aqueous media for enzymatic reactions, the activity of xanthine oxidase in the presence of organic solvents has not received enough attention. The investigation of the oxidative activity of xanthine oxidase in the present study indicated the reduction of the activity in nine aqueous–organic media with methanol and dioxane having the lowest and highest C_{50} values of 2 ± 1 and 15 ± 3%, v/v, respectively. In some cases this reduction occurred in a nonlinear manner. A similar phenomenon has been reported by others with some other enzymatic systems [22, 25-27].

The sharp decrease in the enzyme activity caused by organic solvents could be attributed to an immediate denaturation of enzyme due to destruction of the enzyme hydration shell [28] or a fast reduction in the number of enzyme active sites [25]. Ruth et al. interpreted this phenomenon in terms of conformational changes [22]. It is also possible that the replacement of water molecules from the enzyme causes the unfolding of the enzyme molecule with exposure of the inner important residues to organic solvent resulting in faster denaturation rate [26]. Nevertheless, it is less likely to obtain a comprehensive interpretation for all enzyme–solvent interactions and the effects of the solvent on biocatalytic activity [29].

Taking into account the complexity of the reactions catalyzed by xanthine oxidase [21] and a large flexibility of the active site of this enzyme [30], it is difficult to describe all results with a single model. This explains why a comprehensive and reasonable mechanism for the behavior of xanthine oxidase in organic solvents was not proposed in the previous studies [19-21]. It is likely that in a mixture of buffer solution and a water-miscible

organic solvent as the reaction medium, xanthine oxidase activity is affected by the solvent through two mechanisms: via the interaction of the organic solvent with the water molecules associated with the enzyme structure, and via direct interaction of the solvent as an inhibitor with enzyme leading to changes in the reaction kinetics.

The stability of xanthine oxidase in aqueous–organic mixtures during a 24-h incubation at 37°C was, in some cases, associated with an increase in the activity. It is likely that a more stable and also appropriate protein conformation is adopted in the absence of substrate [1]. It is also possible that in a dehydrated state the conformational rigidity of the enzyme and its resistance to deleterious processes increase [31]. This "organotolerance" property of enzyme could be of great value in helping one use the advantages of aqueous–organic media for enzymes.

The activity of lyophilized xanthine oxidase increased even in such deleterious solvents as methanol. It is possible that following lyophilization, conformational changes of the enzyme are induced and the protein is locked in various conformations in dry organic solvent [32]. As only a few of these will be catalytically active, an increased activity observed for xanthine oxidase in this study may indicate that the contribution of appropriate conformation(s) among the locked conformations is high. Meanwhile, it appears that the water, which was present in the incubation medium, was enough to provide the hydration layer required for the enzyme to manifest its biological activity [33, 34].

In conclusion, some information and evidence were produced that would be useful in the development of reaction conditions for higher activity and stability of xanthine oxidase. The results could find some industrial applications, e.g. in designing and production of new biosensors or production of some xanthine oxidase-derived compounds.

REFERENCES

1. Yoon, J. H., and Mckenzie, D. (2005) *Enzyme Microb. Technol.*, **36**, 439-446.
2. Tsai, S. W., Chen, C. C., Yang, H. S., Ng, I. S., and Chen, T. L. (2006) *Biochim. Biophys. Acta*, **764**, 1424-1428.
3. Castillo, E., and Lopez-Munguia, A. (2004) *J. Biotechnol.*, **114**, 209-217.
4. Schumacher, J., Eckstein, M., and Kragl, U. (2006) *Biotechnol. J.*, **1**, 574-581.
5. Lima, V. M. G., Krieger, N., Mitchell, D. A., and Fontana, J. D. (2004) *Biochem. Eng. J.*, **18**, 65-71.
6. Wu, J. C., Lee, S. S., Mahmood, M. M. B., Chow, Y., Talukder, M. M. R., and Choi, W. J. (2007) *J. Mol. Catal. B: Enzymatic*, **45**, 108-112.
7. Barros, M. R., Carvalho, M. G. V., Garcia, F. A. P., and Pires, E. M. W. (1992) *Biotechnol. Lett.*, **14**, 179-184.
8. Krenitsky, T. A., Neil, S. M., Elion, G. B., and Hitchings, G. H. (1972) *Arch. Biochem. Biophys.*, **150**, 585-599.
9. Krenitsky, T. A., Spector, T., and Hall, W. W. (1986) *Arch. Biochem. Biophys.*, **247**, 108-119.
10. Van Scoik, K. G., Johnson, C. A., and Porter, W. R. (1985) *Drug Metab. Rev.*, **16**, 157-174.
11. Salti, I., and Hemady, K. (1980) *Clin. Chem.*, **26**, 793-794.
12. Yamamoto, T., Moriwaki, Y., Suda, M., Nasako, Y., Takahashi, S., Hiroishi, K., Nakano, T., Hada, T., and Higashino, K. (1993) *Biochem. Pharmacol.*, **46**, 2277-2284.
13. Krenitsky, T. A., Hall, W. W., de Miranda, P., Beauchamp, L. M., Schaeffer, H. J., and Whiteman, P. D. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3209-3213.
14. Halliwell, B., and Cutteridge, M. C. (1999) *Free Radicals in Biology and Medicine*, 3rd Edn., Oxford University Press, New York, pp. 28-30.
15. Ohta, Y., Matsura, T., Kitagawa, A., Tokunaga, K., and Yamada, K. (2007) *Free Radic. Res.*, **41**, 135-144.
16. Nakatani, H. S., Santos, L. V., Pelegrine, C. P., Terezinha, S., Gomes, M., Matsushita, M., Souza, N. E., and Visentainer, J. V. (2005) *Am. J. Biochem. Biotechnol.*, **1**, 85-89.
17. Mao, L., Xu, F., Xu, Q., and Jin, L. (2001) *Anal. Biochem.*, **292**, 94-101.
18. Stredansky, M., Pizzariello, A., Miertus, S., and Svorc, J. (2000) *Anal. Biochem.*, **285**, 225-229.
19. Fridovich, I. (1966) *J. Biol. Chem.*, **10**, 3624-3629.
20. Dastoli, F. R., and Price, S. (1967) *Arch. Biochem. Biophys.*, **118**, 163-165.
21. Dastoli, F. R., and Price, S. (1967) *Arch. Biochem. Biophys.*, **122**, 289-291.
22. Ruth, D. M., Buckley, S. J., O'Connor, B. F., and O'Fagain, C. (2007) *Enzyme Microb. Technol.*, **41**, 307-311.
23. Guo, B. Y., Wen, B., Shan, X. Q., Zhang, S. Z., and Lin, J. M. (2005) *J. Chromatogr. A*, **1074**, 205-213.
24. Miroliaei, M., and Nemat-Gorgani, M. (2002) *Int. J. Biochem. Cell Biol.*, **34**, 169-175.
25. Szabelski, M., Stachowiak, K. K., and Wiczk, W. (2001) *Acta Biochim. Pol.*, **48**, 1197-1201.
26. D'Acunzo, F., Barreca, A. M., and Galli, C. (2004) *J. Mol. Catal. B: Enzym.*, **31**, 25-30.
27. Pazhang, M., Khajeh, K., Ranjbar, B., and Hosseinkhani, S. (2006) *J. Biotechnol.*, **127**, 45-53.
28. Simon, L. M., Kotorman, M., Szabo, A., Nemcsok, J., and Laczko, I. (2007) *Proc. Biochem.*, **42**, 909-912.
29. Barberis, S., Quiroga, E., Morcelle, S., Priolo, N., and Luco, J. M. (2006) *J. Mol. Catal. B: Enzym.*, **38**, 95-103.
30. Beedham, C. (2002) in *Enzyme Systems that Metabolize Drugs and Other Xenobiotics* (Ioannides, C., ed.) John Wiley & Sons Ltd, New York, pp. 147-187.
31. Volkin, D. B., Staubli, A., Langer, R., and Klibanov, A. M. (1991) *Biotechnol. Bioeng.*, **37**, 843-853.
32. Van Unen, D. J., Engbersen, J. F. J., and Reinhoudt, D. N. (2001) *J. Mol. Catal. B: Enzym.*, **11**, 877-882.
33. Gupta, M. N. (1992) *Eur. J. Biochem.*, **203**, 25-32.
34. Gupta, M. N., and Roy, I. (2004) *Eur. J. Biochem.*, **271**, 2575-2583.