Biochemistry (Moscow), Vol. 68, No. 3, 2003, pp. 329-333. Translated from Biokhimiya, Vol. 68, No. 3, 2003, pp. 397-402.

Effects of the Water-Miscible Organic Solvents on Lactoperoxidase Purified from Creek-Water Buffalo Milk

H. Özdemir*, H. I. Hacibeyoglu, and Ö. I. Küfrevioglu

Faculty of Arts and Sciences, Department of Chemistry, Ataturk University, 25240 Erzurum, Turkey; E-mail: hozdemir@atauni.edu.tr

Received February 26, 2002
Revision received May 27, 2002

Abstract—Water buffalo lactoperoxidase (WBLPO) was purified with Amberlite CG-50 (NH_4^+ form) resin, CM-Sephadex C-50 ion-exchange chromatography, and Sephadex G-100 gel-filtration chromatography from skimmed buffalo milk. The purity of the WBLPO was shown with SDS-PAGE. The R_z (A_{412}/A_{280}) value for the WBLPO was 0.9. The optimum pH for the WBLPO was at 6.0. The K_m value at optimum pH and 25°C was 0.13 mM. The V_{max} value at optimum pH and 25°C was 5.3 μmol/min per ml. The K_i values for methanol, ethanol, dimethyl sulfoxide (DMSO), acetonitrile, isopropanol, tetrahydrofuran (THF), N,N′-dimethylformamide (DMF), and ethylene glycol were 1.087, 0.364, 0.302, 0.459, 0.330, 0.126, 0.093, and 2.125 M, respectively. All the solvents showed competitive inhibition. The I_{50} values of methanol, ethanol, dimethyl sulfoxide, acetonitrile, isopropanol, tetrahydrofuran, N,N′-dimethylformamide, and ethylene glycol were 2.910, 0.942, 0.537, 1.320, 0.875, 0.470, 0.405, and 3.920 M, respectively. Ethylene glycol, methanol, acetonitrile, and ethanol have been found to be very promising solvents for performing biocatalytic reactions with LPO in organic media.

Key words: lactoperoxidase, organic solvents, kinetics

Lactoperoxidase (LPO) (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) is a redox enzyme with antibacterial properties found in several biological fluids, like milk, tears, and saliva [1]. LPO has the ability to catalyze the oxidation of halides and pseudohalides such as thiocyanate by hydrogen peroxide to form potent oxidant and bactericidal agents [2]. LPO is an oxidoreductase secreted into milk and plays an important role in protecting the lactating mammary gland and the intestinal tract of newborn infants against pathogenic microorganism [3]. The enzyme catalyzes the oxidation of endogenous thiocyanate (SCN^-) to the antibacterial hypothiocyanate ($OSCN^-$) [4, 5]. LPO consists of a single polypeptide chain containing 612 amino acid residues and its molecular weight is about 85 kD [6, 7]. It contains 15 half-cystine residues and carbohydrate moieties that comprise about 10% of the weight of the molecule [8, 9].

Peroxidases play significant roles in some industrial applications due to their multifunctional reactivities and substrate specificities [10, 11]. Among the number of peroxidase-mediated catalyses, oxidation and dehydrogenation are generally known to be particularly useful. Enzymatic catalyses in organic solvents have opened a field in the biotechnological applications of proteins [12-14]. The ability of some organic solvents to interfere with the physicochemical properties of enzymes is well documented [15]. Several studies have concentrated on the use of physical properties of solvents to predict the catalytic behavior of enzymes in organic solvents [16]. The effects of different water-miscible organic solvents on biocatalytic activities of chloroperoxidase and horseradish peroxidase have been determined [17]. Several researchers have reported the application of peroxidase, especially horseradish peroxidase (HRP), in organic media [18, 19]. HRP is active in water-miscible solvent concentration of 70-90% (v/v), although some enzyme denaturation occurs in 20-50% (v/v) of water miscible solvents [20-23].

In this work we purified WBLPO enzyme from domestic creek-water buffalo milk and investigated its kinetic properties. Inhibition kinetics of the WBLPO in water-miscible organic solvents has not been previously reported. We report WBLPO activity and kinetic constant (K_i and I_{50} values) in water-miscible organic solvents (methanol, ethanol, dimethyl sulfoxide, acetonitrile, isopropanol, tetrahydrofuran, N,N′-dimethylformamide, and ethylene glycol) in *in vitro*.

Abbreviations: LPO) lactoperoxidase; DMSO) dimethylsulfoxide; THF) tetrahydrofuran; DMF) N,N′-dimethylformamide; ABTS) 2,2′-azinobis(3-ethylbenzthiazolinesulfonate) diammonium salt.

* To whom correspondence should be addressed.

0006-2979/03/6803-0329$25.00 ©2003 MAIK "Nauka/Interperiodica"

MATERIALS AND METHODS

Purification of LPO. Buffalo milk was centrifuged at 2500 rpm at 4°C for 15 min to remove fat. Amberlite CG-50 (NH_4^+) resin (equilibrated with 5 mM sodium acetate, pH 6.8) was added at the rate of 22 g/liter to the fresh raw skimmed milk and stirred for 1 h [4, 24]. The supernatant was decanted. The resin was washed with distilled water and 20 mM sodium acetate (pH 6.8). The bound protein was eluted with 0.5 M sodium acetate, pH 6.8. To the green-colored mixture solid ammonium sulfate was gradually added (first precipitation, 90% saturation) over a period of 30 min and the enzyme solution was dialyzed overnight against 5 mM sodium phosphate buffer, pH 6.8.

The clear greenish supernatant as obtained above was loaded onto a column of CM-Sephadex C-50 (Fluka, Germany) (3 × 10 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column-bound enzyme was washed with 100 ml of 10 mM phosphate buffer, pH 6.8, containing 100 mM NaCl. The enzyme was eluted with a linear gradient of 100-200 mM NaCl in 10 mM phosphate buffer, pH 6.8, and subjected to a second ammonium sulfate precipitation (90% saturation), then the enzyme solution was dialyzed overnight against 5 mM sodium phosphate buffer, pH 6.8.

The dialyzed LPO sample as obtained above was applied to a column of Sephadex G-100 (Fluka) (2.5 × 100 cm). The column-bound enzyme was eluted with 0.1 M phosphate buffer, pH 6.8, and salted out with 90% saturation of a third ammonium sulfate precipitation. The enzyme solution was dialyzed overnight against 0.5 M sodium phosphate buffer, pH 6.0. Fractions were lyophilized and checked for purity by SDS-PAGE gel [25]. Protein concentration was determined according to the Lowry method [26].

Measurement of WBLPO activity. Lactoperoxidase activities were determined by the procedure of Shindler with a slight modification [27]. This method is based on oxidation of 2,2′-azino-bis(3-ethylbenzthiazolinesulfonate) diammonium salt (ABTS) as a chromogenic substrate by means of H_2O_2, and colored compound, which occurs during the reaction and gives absorbance at 412 nm. Briefly, 2.8 ml of 1 mM ABTS in phosphate buffer (0.1 M, pH 6.0) was mixed with 0.1 ml of the enzyme in 1 mM phosphate buffer, pH 6.8, and 0.1 ml of 3.2 mM H_2O_2 solution. The absorbance was taken at 412 nm as a function of time every 15 sec. One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ABTS per 1 min at 25°C (molar absorption coefficient, 32,400 $M^{-1}\cdot cm^{-1}$).

SDS-polyacrylamide gel electrophoresis. After purification of the enzyme, SDS-PAGE was carried out according to the Laemmli method [25]. This was done in 4 and 10% acrylamide concentration for stacking and running gel, respectively, containing 0.1% SDS. Protein standards of known molecular weight are electrophoresed and their mobilities measured and plotted as a function of the log of the molecular weight [28].

Determination of WBLPO activity in organic solvents. Water-miscible organic solvents such as methanol, ethanol, dimethyl sulfoxide, acetonitrile, isopropanol, tetrahydrofuran, N,N′-dimethylformamide, and ethylene glycol were selected for screening of WBLPO activity. To obtain K_i values at pH 6.0, the enzyme activity was measured for six different substrates concentrations at 25°C by measuring absorbance at 412 nm. In the media with or without solvent the substrate concentrations were $6.66\cdot10^{-2}$, $10.0\cdot10^{-2}$, $16.7\cdot10^{-2}$, $26.6\cdot10^{-2}$, $36.6\cdot10^{-2}$, and $50.0\cdot10^{-2}$ mM. Solvent concentrations in the reaction medium are given in Table 2. K_i values were calculated from Lineweaver–Burk graphs, and average K_i values were calculated for each solvent.

To determine I_{50} values of the solvents, the enzyme activity was measured at 25°C at 412 nm. Percent activity values were obtained from six different solvent concentrations with constant substrate concentration. LPO activity without solvent was taken as 100%. The inhibitor concentrations causing 50% inhibition (I_{50}) by the solvents were calculated from the activity–solvent concentration graphs.

RESULTS AND DISCUSSION

WBLPO was eluted from CM-Sephadex C-50 ion-exchange chromatography and the R_z (A_{412}/A_{280} nm) values of the fractions were measured. Fractions having R_z value 0.7 or higher were pooled. The enzyme obtained from ion-exchange chromatography was applied to Sephadex G-100 gel filtration chromatography. Fractions having R_z value 0.7 or higher were pooled. The purity of WBLPO was monitored by SDS-PAGE (Fig. 1). As shown in Fig. 1, WBLPO has a molecular weight of about 85 kD. The WBLPO has the same band position as purified LPO from other sources [3].

As shown in Table 1, specific activity was calculated for crude extract and purified enzyme solution, yielding a purification of 13.2-fold and yield of 7.54 mg (R_z = 0.9) from 1 liter of buffalo milk. The yields of lactoperoxidase from buffalo milk and cow milk were reported to be 10 and 9 mg/liter, respectively [4]. Kinetic parameters of WBLPO, such as optimum pH, optimum temperature, K_m, and V_{max}, were determined using ABTS as substrate. The pH optimum determined from an activity–pH plot was 6.0. Optimum temperature at optimum pH was 60°C. K_m and V_{max} values at 25°C were determined from Lineweaver–Burk plots using $1/v$–$1/[S]$ values as shown in Fig. 2. K_m value at optimum pH and 25°C was 0.13 mM; V_{max} at optimum pH and 25°C was 5.3 μmol/min per ml.

Inhibition kinetics of WBLPO in water-miscible organic solvent has not been reported previously. LPO

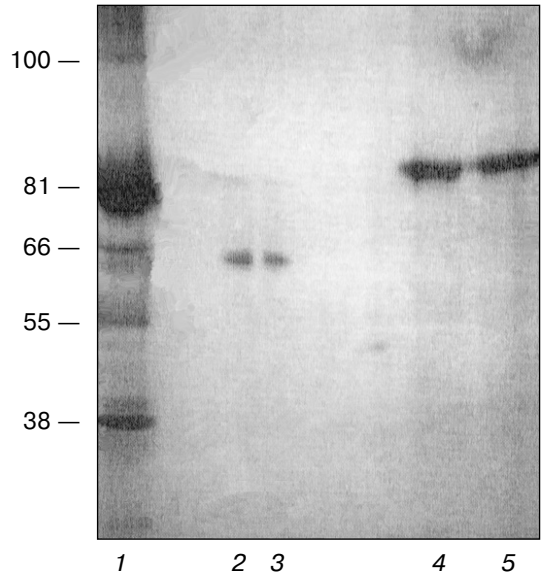

Fig. 1. SDS-PAGE of lactoperoxidase: *1*) protein standards (yeast hexokinase, rabbit heart creatine phosphokinase, bovine serum albumin, bovine liver glutamate dehydrogenase, bovine spleen deoxyribonuclease); their molecular mass values are given on the left in kD; *2*, *3*) rainbow trout glucose 6-phosphate dehydrogenase; *4*, *5*) purified buffalo milk lactoperoxidase.

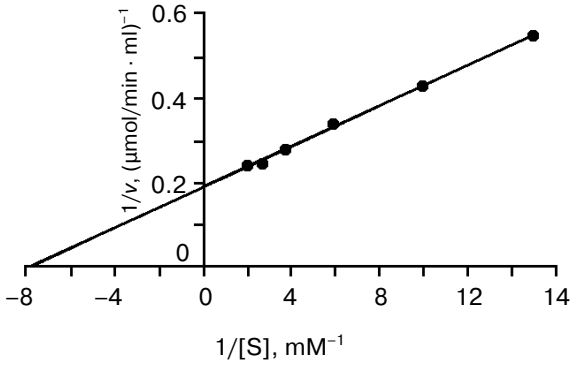

Fig. 2. Dependence of LPO activity on ABTS concentration in Lineweaver–Burk coordinates at pH 6.0 and 25°C.

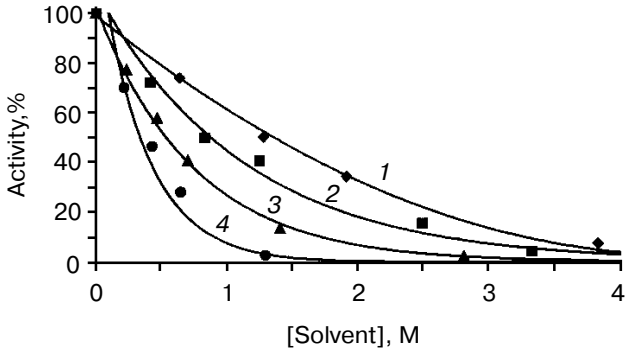

Fig. 3. Dependence of LPO activity (%) on solvent concentration: *1*) acetonitrile; *2*) isopropanol; *3*) DMSO; *4*) DMF. ABTS concentration, $26.64 \cdot 10^{-2}$ mM.

activity in water-miscible organic solvents (methanol, ethanol, dimethyl sulfoxide, acetonitrile, isopropanol, tetrahydrofuran, N,N′-dimethylformamide, and ethylene glycol) in *in vitro* was investigated and kinetic constants (K_i and I_{50} values) were reported. To show inhibition effects, the most suitable parameters are the K_i and I_{50} values. Therefore, in this study, both K_i and I_{50} parameters of these water-miscible organic solvents for WBLPO activity were determined. As shown Table 2, K_i values were calculated from Lineweaver–Burk plots. The K_i values of methanol, ethanol, dimethyl sulfoxide, acetonitrile, isopropanol, tetrahydrofuran, N,N′-dimethylformamide, and ethylene glycol were 1.087, 0.364, 0.302, 0.459, 0.330, 0.126, 0.093, and 2.126 M, respectively. In addition to K_i values, types of inhibition by the solvents were obtained from Lineweaver–Burk plots. All the solvents show competitive inhibition. As shown in Table 2 and Figs. 3 and 4, the inhibitor concentrations causing 50% inhibition were determined from activity–solvent concentration plots. The obtained I_{50} values for methanol, ethanol, dimethyl sulfoxide, acetonitrile, isopropanol, tetrahydrofuran, N,N′-dimethylformamide, and ethylene glycol were 2.910, 0.942, 0.537, 1.320, 0.875, 0.470, 0.405, and 3.920 M, respectively. As evident from I_{50} values, LPO inhibition in ethylene glycol is lower than in methanol, acetonitrile, ethanol, isopropanol,

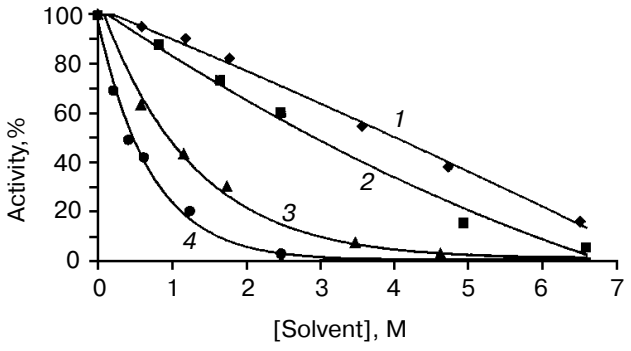

Fig. 4. Dependence of LPO activity (%) on solvent concentration: *1*) ethylene glycol; *2*) methanol; *3*) ethanol; *4*) THF. ABTS concentration, $26.64 \cdot 10^{-2}$ mM.

Table 1. Stages of purification of lactoperoxidase from domestic creek-water buffalo milk

Step	Activity, U/ml	Volume, ml	Protein concentration, mg/ml	Total protein, mg	Total activity, U/ml	Specific activity, U/mg	Yield, %	Purification degree
Crude homogenate	3.47	170	1.98	336.6	585.9	1.74	100	1.00
Ammonium sulfate precipitation	17.1	30	2.01	60.3	513	8.5	87	4.88
CM-Sephadex C-50 column	1.5	312	0.12	37.44	468	12.5	79	7.18
Ammonium sulfate precipitation	13.5	30	0.78	23.4	405	17.3	69	9.94
Sephadex G-100 column	1.6	170	0.09	15.3	272	17.7	46	10.17
Ammonium sulfate precipitation and dialysis	6.01	29	0.26	7.54	174.2	23.1	29	13.2

Table 2. Values of K_i obtained from Lineweaver–Burk plots for lactoperoxidase (LPO) in the presence of three different concentrations of the solvent (I_{50} values were determined from dependences of LPO activity on solvent concentration at the constant substrate concentration of $26.64 \cdot 10^{-2}$ mM)

Solvent	[Solvent], M	K_i, M	Mean ± SD (K_i, M)	Inhibition type	Inhibition, %	I_{50}, M
Methanol	0.823	1.110	1.087 ± 0.050	competitive	12.5	2.910
	1.646	1.121			17.1	
	2.468	1.030			40.3	
Ethanol	0.578	0.374	0.364 ± 0.015	competitive	36.9	0.942
	1.156	0.371			56.7	
	1.734	0.347			70.0	
DMSO	0.235	0.310	0.302 ± 0.008	competitive	22.8	0.537
	0.469	0.300			42.3	
	0.704	0.295			59.2	
Acetonitrile	0.639	0.464	0.459 ± 0.024	competitive	26.0	1.320
	1.278	0.433			49.8	
	1.917	0.481			65.6	
Isopropanol	0.417	0.334	0.330 ± 0.022	competitive	28.0	0.875
	0.834	0.306			51.2	
	1.251	0.350			59.3	
THF	0.206	0.118	0.126 ± 0.0121	competitive	31.0	0.470
	0.411	0.109			51.0	
	0.616	0.150			58.0	
DMF	0.217	0.110	0.093 ± 0.021	competitive	30.0	0.405
	0.433	0.098			54.6	
	0.650	0.070			71.9	
Ethylene glycol	0.591	2.178	2.126 ± 0.049	competitive	10.0	3.920
	1.182	2.119			12.0	
	1.773	2.080			46.0	

DMSO, THF, and DMF, respectively. I_{50} values correlate with the K_i values of the solvents as shown in Table 2.

In conclusion, WBLPO has been purified from the domestic creek-water buffalo milk and the purified WBLPO activity measured in water-miscible solvents. K_m and V_{max} values have been determined. Ethylene glycol was found to be most effective solvent in which the enzyme shows high activity, while WBLPO activity decreases in the series methanol, acetonitrile, ethanol, isopropanol, dimethylsulfoxide, tetrahydrofuran, and N,N′-dimethylformamide, respectively.

REFERENCES

1. Dumonte, C., and Rousst, B. (1983) *J. Biol. Chem.*, **258**, 14166-14172.
2. Reiter, B., and Perrandin, J. P. (1991) in *Peroxidase in Chemistry and Biology* (Everse, J., Everse, K. E., and Grisham, M. B., eds.) Vol. 1, CRC Press, Boca Raton, FL, USA, pp. 143-180.
3. Ueda, T., Sakamaki, K., Kuroki, T., Yano, I., and Nagata, S. (1976) *Eur. J. Biochem.*, **243**, 32-41.
4. Kumar, R., and Bhatla, K. L. (1995) *Acta Cryst.*, **D51**, 1094-1096.
5. Hamon, C. B., and Klebanoff, S. I. (1973) *J. Exp. Med.*, **134**, 438-441.
6. Cals, M., Maillierat, P., Birignon, G., Anglade, P., and Dumas, B. R. (1991) *Eur. J. Biochem.*, **198**, 733-739.
7. Paul, K. G., and Ohlsson, P. I. (1985) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., and Tenovue, Y.O., eds.) Vol. 27, Marcel Dekker Inc., N. Y., pp. 15-29.
8. Dewil, J. N., and van Hooydonk, A. C. M. (1996) *Netherlands Milk Dairy*, **50**, 227-244.
9. Caristrom, A. (1969) *Biochim. Biophys. Acta*, **23**, 185-202.
10. D'Ischia, M., Napolitano, A., and Prota, G. (1991) *Biochim. Biophys. Acta*, **1073**, 423-430.
11. De Montellano, P. R. O., and Crab, L. A. (1987) *Biochemistry*, **26**, 5310-5314.
12. Zaks, A., and Klibanov, A. M. J. (1988) *J. Biol. Chem.*, **263**, 3194.
13. Khmelnistky, Y. L., Levashov, A. V., Klyachko, N. L., and Martinek, K. (1988) *Enzyme Microb. Technol.*, **10**, 710-718.
14. Dordick, J. S. (1989) *Enzyme Microb. Technol.*, **11**, 194.
15. Klibanov, A. M. (1989) *Chemtech.*, **16**, 354-359.
16. Halling, P. J. (1994) *Enzyme Microb. Technol.*, **16**, 178.
17. Torres, E., Siminiovich, B., Barzana, E., and Vazquez-Duhalt, R. (1998) *J. Mol. Catal.*, **B4**, 155-159.
18. Takahashi, K., Nishimura, H., Yoshimito, T., Saito, Y., and Inada, Y. (1984) *Biochem. Biophys. Res. Commun.*, **121**, 261-265.
19. Mabrouk, P. A. J. (1995) *Am. Chem. Soc.*, **117**, 2141-2146.
20. Dordick, J. S., Marietta, M. A., and Klibanov, A. M. (1987) *Biotechnol. Bioeng.*, **30**, 31-36.
21. Propp, J. L., Kirk, T. K., and Dordick, J. S. (1991) *Enzyme Microb. Technol.*, **13**, 964-968.
22. Ryu, K., and Dordick, J. S. (1990) *Res. Conserv. Recycl.*, **3**, 177-185.
23. Ryu, K., and Dordick, J. S. (1992) *Biochemistry*, **31**, 2588-2598.
24. Özdemir, H., Aygül, I., and Küfrevioglu, Ö. I. (2001) *Prep. Biochem. Biotech.*, **31**, 125-134.
25. Laemmli, U. K. (1970) *Nature* (London), **227**, 680-685.
26. Segel, I. H. (1968) *Biochemical Calculations*, John Wiley and Sons, Inc., New York, p. 403.
27. Shindler, J. S., and Bardsley, W. G. (1975) *Biochem. Biophys. Res. Commun.*, **67**, 1307-1312.
28. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.*, **244**, 4406.