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Reaction of manganese-dependent peroxidase from *Bjerkandera adusta* in aqueous organic media

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Abstract

The oxidation of various phenolics and aromatic amines by manganese-dependent peroxidase (MnP) of *Bjerkandera adusta* was examined in aqueous organic media. MnP retained its activities in several 70% (v/v) aqueous solutions of water-miscible organic solvents including ethylene glycol, diethylene glycol, acetone and acetonitrile. The absorption spectra of MnP in these aqueous organic media were similar to that observed in the reaction without solvent addition, indicating that the heme of MnP was little affected by the addition of these water-miscible organic solvents. MnP was also found to oxidize Mn(II) to Mn(III) in these 70% (v/v) aqueous organic media. The oxidation of Mn(II) by MnP was correlated with the Dimroth–Reichardt parameter, $E_T(30)$, of the solvents. Furthermore, MnP catalyzed the oxidation of anisidines, aminophenols, phenylenediamines and phenolics in aqueous 70% (v/v) acetone, acetonitrile and diethylene glycol media. Aromatic amines that have high hydrophobicity were shown to be suitable for the reaction of MnP in aqueous water-miscible organic media.

Keywords: *Bjerkandera adusta*; Manganese-dependent peroxidase; Organic solvents; Lignin-degrading enzyme; Radical oxidation

1. Introduction

Ligninolytic enzymes, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) [1–3] have been found in culture filtrates of various white-rot basidiomycetes including *Phanerochaete chrysosporium* [4], *Coriolus versicolor* [5], *Phlebia radiata* [6], *Bjerkandera adusta* [7], *Pleurotus ostreatus* [8] and *Lentinus edodes* [9]. LiP and MnP contain one mole of iron protoporphyrin IX per mole of the enzyme. LiP catalyzes H_2O_2-dependent one-electron oxidation of a variety of non-phenolics to generate aryl cation radicals [4,10]. These radicals undergo subsequent nonenzymatic reactions to yield a variety of final products. MnP is also an H_2O_2-dependent enzyme and oxidizes a variety of dyes, phenolic compounds and amines [11]. This enzyme is known to oxidize Mn(II) to Mn(III) which acts as a mediator and further oxidizes aromatic substrates [12].

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Interestingly, these enzymes have also been found to catalyze the oxidation of halogenated phenolic compounds, polycyclic aromatic hydrocarbons (PAH) and other compounds which are resistant to microbial attack [2,13]. Therefore, the ligninolytic enzymes can be applicable to the removal of toxic chemicals such as phenanthrene, benzopyrene and dioxins from soil and water polluted with these compounds [14–16].

However, most of the substrates for these enzymes are scarcely or less soluble in water. Therefore, the development of a reaction system using organic solvents as the reaction media is necessary for the degradation and transformation of these aromatic compounds. It has become clear that many enzymes can function in organic solvents as well as in water [17–23]. LiP was also shown to be able to catalyze the oxidation of 3,3′-dimethoxybenzidine [24,25] and PAH [26] in the presence of organic solvents. Recently, MnP was shown to be highly tolerant to organic solvents compared to LiP [27] and to be able to oxidize PAH [28] and lignin [29] in acetone. However, the effects of organic solvents on the catalytic activity and substrate specificity of MnP are not well understood.

This research was carried out to test the possibility of using MnP in organic solvents. It aims also to find the relationship among enzyme protein, substrates and solvents in the reaction of MnP in organic solvents.

2. Materials and methods

2.1. Purification of MnP

MnP was purified from the culture filtrates of *B. adusta* (K-2679) grown at 30°C in 200-ml Erlenmeyer flasks with 15 ml of culture medium containing 2% (w/v) glucose, 0.5% (w/v) polypeptone, 0.2% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4 and 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ as described by Kimura et al. [7]. MnP activity was determined by the method of Kofujita et al. using guaiacol as a substrate [8]. One unit of MnP activity was defined as the amount of enzyme which increased absorbance by 1.0 per minute. The protein concentration was determined by the method of Bradford [30] using bovine serum albumin as a standard. Purification of enzyme was carried out using DEAE-Sepharose CL-6B and Mono-Q ion-exchange columns (Amersham Pharmacia Biotech, Sweden) by the method of Kirk et al. [31]. The purified MnP had a pI of 2.8 and a molecular weight of 42 000.

2.2. Measurement of MnP activity in the presence of organic solvents

The rates of oxidation of substrates by MnP in the presence of water-miscible and water-immiscible organic solvents were measured by the modified method of Kofujita et al. [8] using a reaction mixture containing 14 mM substrate, 50 mM Na lactate (pH 4.5), 0.2 mM $MnSO_4$, 0.25 mM H_2O_2, organic solvent to give a 70% (v/v) concentration and the enzyme solution in a final volume of 1 ml. Lactic acid was used instead of Na lactate in the reaction in water-immisible solvents because of the insolubility of Na lactate in these solvents. The reaction was started by adding H_2O_2. The reaction rate was estimated from the increase in absorbance at λ_{max} of the oxidation products. For the reaction in water-immiscible organic solvents, MnP solution was first dried by rotary evaporator at 30°C and the solvent was added to prepare MnP stock solution. In this case, MnP was dispersed in the solvents to make homogeneous enzyme suspensions. No decrease of MnP activity was observed during this operation. Immediately after the preparation of the enzyme, 0.7 ml of solution containing 0.57 mM substrate, 71.4 mM lactic acid and 0.29 mM $MnSO_4$ dissolved or suspended in each organic solvent was added to an aliquot of MnP stock solution. The pH of the reaction mixture was not adjusted in this study.

The reaction was initiated by the addition of 0.3 ml of the organic solvent saturated with H_2O_2 [32]. The reaction was monitored by measuring the increase in absorbance at λ_{max} of the reaction products. The substrates and λ_{max} of the reaction products are listed in Table 5. MnP activity was calculated by subtracting the activity determined in the absence of Mn(II) from that in the presence of Mn(II). The change of absorbance without added Mn(II) represented the activity of Mn(II)-independent peroxidase. One unit of the activity was defined as an increase in absorbance of 1.0 per minute.

2.3. Oxidation of Mn(II) by MnP in aqueous organic media

The oxidation of Mn(II) by MnP in 70% (v/v) aqueous water-miscible organic media was evaluated spectrophotometrically using a reaction mixture containing 50 mM Na lactate (pH 4.5), 0.2 mM $MnSO_4$ as a substrate, 0.1 mM H_2O_2, 70% (v/v) organic solvent and the enzyme solution in a final volume of 1 ml. In the reaction in water, the generation of Mn(III) is monitored at 240 nm by the production of Mn(III)-lactate complex [8,33]. However, acetone, dioxane, *N*,*N*′-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and pyridine have intense absorbance at around 240 nm, which interferes with the estimation. Therefore, the activity was measured at 340 nm. By measuring the absorbance at this wavelength the influence of the solvents was minimized. One unit of MnP activity was defined as the amount of enzyme which increased the absorbance by 1.0 per minute.

2.4. Non-enzymatic oxidation of guaiacol by Mn(III)

Oxidation of guaiacol by Mn(III) was monitored spectrophotometrically at 465 nm using a reaction mixture containing 0.8 mM guaiacol, 0.2 mM Mn(III)-lactate complex and 70% (v/v) organic solvent in a final volume of 1 ml. An Mn(III)-lactate complex was prepared by disolving Mn(III) acetate in 1.0 M Na lactate (pH 4.5) to give a concentration of 4 mM.

2.5. Measurement of absorption spectra

Absorption spectra of MnP in the solution containing 70% (v/v) organic solvents were recorded on a Shimadzu UV-160A spectrophotometer.

3. Results

3.1. Reaction of MnP in the presence of organic solvents

Activities of MnP in 70% (v/v) aqueous water-miscible and water-immiscible organic solvents were measured using guaiacol, 2,6-dimethoxyphenol and 3,3′-dimethoxybenzidine as substrates. As shown in Table 1, in the enzymatic reaction using guaiacol as a substrate, the activity of MnP in 70% (v/v) aqueous acetone and acetonitrile media was 44% and 60% of that observed in the aqueous buffer (without addition of the solvent), respectively. In the case of 2,6-dimethoxyphenol oxidation, activities in aqueous acetone, diethylene glycol dimethyl ether (DEGDE) and 1,2-dimethoxyethane media were found to be slightly lower than that observed in water. When 3,3′-dimethoxybenzidine was a substrate, MnP activities in aqueous acetone, acetonitrile and diethylene glycol media were 168%, 118% and 219%, respectively, of the activity in water. However, addition of DMF, DMSO, methanol, pyridine and tetrahydrofuran (THF) resulted in a remarkable loss of activity in the oxidation of these substrates.

The structural change of MnP caused by the addition of solvents will correlate to the loss of the activity. The UV/VIS spectra of MnP in 70% (v/v) solvents were recorded. As shown in Table 2, the spectrum of MnP had an absorption maximum at 407 nm with smaller peaks at 503 and 642 nm in aqueous buffer. Absorption maxima at around 407 nm were observed in 70%

Table 1
Activity of MnP in 70% (v/v) aqueous organic media
2,6-DMP, 2,6-dimethoxyphenol; 3,3′-DMB, 3,3′-dimethoxybenzidine; DEGDE, diethylene glycol dimethyl ether; DEGME, diethylene glycol monomethyl ether; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; (–), not determined.
Specific activity in the reaction system without addition of the solvent: for guaiacol, 404 U/mg; for 2,6-DMP, 1987 U/mg, for 3,3′-DMB, 518 U/mg.

Solvents	Relative activity (%)		
	Guaiacol	2,6-DMP	3,3′-DMB
Water	100	100	100
70% Acetone	44	82	168
70% Acetonitrile	60	50	118
70% DEGDE	17	94	53
70% DEGME	18	36	0
70% Diethylene glycol	18	18	219
70% 1,2-Dimethoxyethane	23	90	51
70% Dioxane	7	11	1
70% DMF	0	0	0
70% DMSO	0	0	0
70% Ethanol	9	0	0
70% Ethylene glycol	15	12	15
70% Methanol	0	0	0
70% Methylcellosolve	15	12	33
70% 1-Propanol	16	5	36
70% 2-Propanol	29	13	7
70% Pyridine	0	0	0
70% THF	1	0	0
Benzene	0	–	0
Chloroform	0	–	0
Ethyl acetate	0	–	0
Toluene	0	–	0

(v/v) aqueous acetone, acetonitrile, ethylene glycol, methylcellosolve and diethylene glycol, indicating that the heme of MnP was little affected by the addition of these organic solvents. In fact, MnP activity was retained in the presence of these solvents as shown in Table 1. It was also confirmed that the spectral properties of the reaction products were not changed by the addition of the solvents used (data not shown). Although MnP also showed the activity in aqueous DEGDE, diethylene glycol monomethyl ether (DEGME), 1,2-dimethoxyethane and 1- and 2-propanol media, absorption spectra of MnP in the presence of these solvents were markedly different from a typical absorption spectrum in the system without addition of the solvent.

Another factor affecting the activity was change of pH of the reaction mixture by the addition of solvents. MnP shows maximum activity at around pH 4.5 [34] in water. Addition of water-miscible solvents was found not to cause a change in the reaction mixture. However, addition of pyridine shifted the pH from 4.5 to 7.7. Chloroform, on the other hand, lowered the pH to 2.5 and benzene, toluene and ethyl acetate changed it to around 4.0. It was already found that at pH 4.0 MnP activity was lowered to 40% of that at pH 4.5. At pH 3.0 and 8.0, the activity fell to below 5% of the highest level. Therefore, water-immiscible solvents caused the decrease in MnP activity by changing pH of the reaction mixture, whereas water-miscible solvents did not effect the activity.

Table 2
Spectral characteristics of MnP in 70% (v/v) aqueous water-miscible organic media
DEGDE, diethylene glycol dimethyl ether; DEGME, diethylene glycol monomethyl ether; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

Solvents	Absorption maxima (nm) [ε (mM^{-1} cm^{-1})]			
Water	407 [123]	503 [16]	642 [5]	
70% Acetone	407 [350]	489 [64]	628 [33]	
70% Acetonitrile	407 [356]	490 [66]	630 [35]	
70% DEGDE	401 [92]	543 [17]	556 [16]	642 [5]
70% DEGME	408 [100]	552 [18]	652 [11]	
70% Diethylene glycol	405 [172]	502 [17]	639 [7]	
70% 1,2-Dimethoxyethane	407 [107]	510 [17]	627 [7]	
70% Dioxane	409 [136]	526 [25]	645 [13]	
70% DMF	403 [184]	502 [18]	639 [8]	
70% DMSO	403 [234]	497 [16]	635 [8]	
70% Ethanol	403 [233]	500 [20]	643 [6]	
70% Ethylene glycol	405 [170]	506 [20]	643 [9]	
70% Methanol	400 [253]	498 [16]	642 [5]	
70% Methylcellosolve	407 [192]	509 [17]	638 [8]	
70% 1-Propanol	406 [236]	504 [39]	532 [35]	644 [10]
70% 2-Propanol	409 [120]	538 [16]	552 [15]	652 [7]
70% Pyridine	410 [220]	530 [21]	556 [19]	
70% THF	406 [208]			

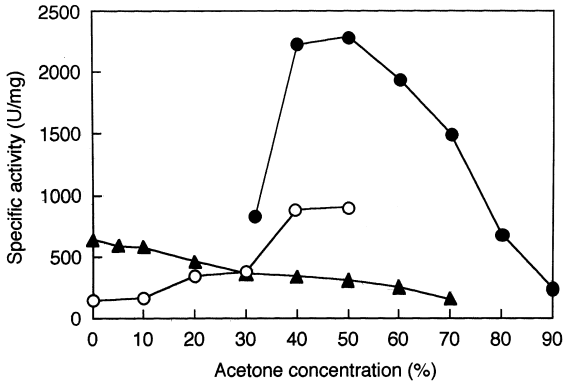

Fig. 1. Effect of acetone concentration on MnP. The activity was measured using guaiacol (-▲-), 3,3′-dimethoxybenzidine dihydrochloride (-○-) and 3,3′-dimethoxybenzidine (-●-) as the substrates by the method described in Materials and methods.

As shown in Table 1, the oxidation of 3,3′-dimethoxybenzidine was remarkably enhanced in 70% acetone. The effect of the acetone concentration on the oxidation of three substrates was estimated. As shown in Fig. 1, the oxidation of 3,3′-dimethoxybenzidine or its dihydrochrolide salt was greatest in 40% to 50% acetone. The activity for guaiacol decreased according to the increase in acetone concentration.

Table 3
Effect of organic solvents on the oxidation of Mn(II) by MnP
DEGDE, diethylene glycol dimethyl ether; DEGME, diethylene glycol monomethyl ether; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; n.l., not listed.
Specific activity in the reaction system without addition of the solvent: 1168 U/mg.

Solvents	Relative activity (%)	$E_T(30)$ (kcal/mol)
Water	100	63.1
70% Acetone	82	42.2
70% Acetonitrile	60	46.0
70% DEGDE	110	38.6
70% Diethylene glycol	18	53.8
70% 1,2-Dimethoxyethane	62	38.2
70% Dioxane	0	36.0
70% DMF	0	43.8
70% DMSO	0	n.l.
70% Ethanol	0	51.9
70% Ethylene glycol	10	56.3
70% Methanol	0	55.5
70% Methyl-cellosolve	1	52.3
70% Pyridine	0	40.2

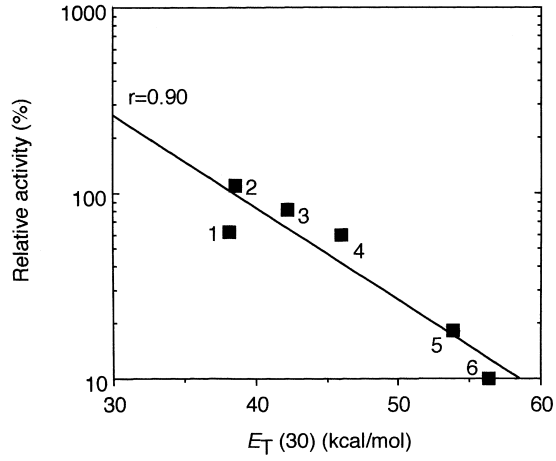

Fig. 2. Relationship between the oxidation of Mn(II) by MnP and solvent polarity, $E_T(30)$. The correlation coefficient was 0.90. The values of $E_T(30)$ of 1,2-dimethoxyethane (1), diethylene glycol dimethyl ether (2), acetone (3), acetonitrile (4), diethylene glycol (5) and ethylene glycol (6) were taken from Ref. [35] and are listed in Table 3.

3.2. Oxidation of Mn(II) to Mn(III) by MnP and non-enzymatic oxidation of the substrates by Mn(III) in aqueous organic media

In the catalytic cycle of MnP, Mn(II) is the primary substrate of MnP, generating Mn(III)

Table 4
Oxidation of guaiacol by Mn(III) in 70% (v/v) aqueous organic media
Activity in water was 0.046 U. DEGDE, diethylene glycol dimethyl ether; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide.

Solvent	Relative activity (%)
Water	100
70% Acetone	87
70% Acetonitrile	183
70% DEGDE	41
70% Diethylene glycol	111
70% 1,2-Dimethoxyethane	37
70% Dioxane	15
70% DMF	15
70% DMSO	22
70% Ethanol	174
70% Ethylene glycol	160
70% Methanol	326
70% Methylcellosolve	67
70% Pyridine	0

by one electron oxidation. Therefore, the correlation between reaction media and the activity for oxidation of Mn(II) to Mn(III) by MnP was estimated. As summarized in Table 3 (column of relative activity), in the reaction in DEGDE, the MnP activity level was higher than that in water. Activity was observed in aqueous acetone, acetonitrile and 1,2-dimethoxyethane media. It was noticeable that the activity for oxidation of Mn(II) coincided with that for oxidation of the three substrates shown in Table 1.

For the solvents which gave the activity, the oxidation activities for Mn(II) of MnP were found to correlate with the Dimroth–Reichardt parameter $E_T(30)$ (Fig. 2) which is directly related to the free energy of the solvation process of the solvents [35]. This experiment showed that the activity to generate Mn(III) from Mn(II) was greater in the solvent which has a low $E_T(30)$ value. This would indicate that solvents which have more energy to solvate with Mn(II) produce less MnP activity. In other words, a higher level of MnP activity resulted from reactions in solvents in which little Mn(II) was replaced by water.

Mn(III) generated by MnP from Mn(II) acts as a strong oxidant to withdraw one electron from the substrate. Therefore, the non-enzymatic oxidation of guaiacol by Mn(III) in 70% aqueous organic media was examined. As shown in Table 4, strong guaiacol-oxidizing activity was observed in aqueous acetonitrile, diethylene glycol and ethylene glycol media. In contrast to the results shown in Table 3, ethanol and methanol promoted the oxidation of guaiacol. This probably indicated that these alcohols

Table 5
Substrate specificities of MnP in water and 70% (v/v) aqueous organic media
HQME, hydroxyquinone monomethyl ether; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). (–), unknown.

Solvents	λ_{max}[a] (nm)	MnP activity in water (U/mg)	Relative activity (%)[b]			Log (Co/Cw)
			Acetone	Acetonitrile	Diethylene glycol	
3,3′-Dimethoxybenzidine	444	518	168	118	219	1.58
o-Phenylenediamine	440	614	0	102	134	0.00
p-Phenylenediamine	459	842	42	46	61	−0.30
m-Phenylenediamine	400	98	33	20	84	−0.40
Aniline	705	0	0	0	0	0.77
o-Anisidine	500	152	20	8	16	0.78
p-Anisidine	550	696	3	12	8	0.45
m-Anisidine	400	18	111	89	67	1.44
o-Aminophenol	440	434	106	84	58	0.47
p-Aminophenol	440	0	0	0	0	0.07
m-Aminophenol	440	86	42	28	23	0.24
Phenol	399	132	9	15	3	1.65
Catechol	398	106	45	57	42	0.91
Hydroquinone	246	930	n.d.	108	30	0.36
Resorcinol	320	88	n.d.	125	45	0.85
Pyrogallol	300	504	n.d.	13	7	0.12
1,2,4-Benzenetriol	471	0	0	0	0	0.35
Guaiacol	465	404	44	60	18	1.01
HQME	371	0	0	0	0	1.50
3-Methoxyphenol	320	0	0	0	0	1.41
2,6-Dimethoxyphenol	468	1987	82	50	18	0.24
Vanillyl alcohol	310	0	n.d.	0	0	0.22
Veratryl alcohol	310	0	n.d.	0	0	0.51
ABTS	415	1468	5	5	22	–

[a] The activities were estimated from the increase in absorbance at λ_{max} of oxidation products given in the table.
[b] [MnP activity (U/mg) in 70% aqueous organic media/MnP activity (U/mg) in the reaction system without addition of the solvent] × 100.

did not interfere with the oxidative activity of Mn(III).

These results suggested that organic solvents affected markedly the enzymatic oxidation of Mn(II) to form Mn(III), which in turn affected the oxidation of the aromatic substrates.

3.3. Substrate specificity of MnP in aqueous organic media

The oxidation of various organic compounds which have been used as substrates for laccase and peroxidase was examined in 70% (v/v) aqueous acetone, acetonitrile and diethylene glycol. Table 5 shows the MnP activities in the presence and absence of the organic solvents. Several aromatic compounds, anisidines, phenylenediamines and phenols tested were oxidized by MnP either in the presence or absence of organic solvents. However, aniline, *p*-aminophenol, 1,2,4-benzenetriol, hydroquinone monomethyl ether (HQME), 3-methoxyphenol, vanillyl alcohol and veratryl alcohol were not oxidized by MnP. In 3,3′-dimethoxybenzidine oxidation, activities in all the aqueous organic media tested were found to be higher than that observed in the reaction without addition of the solvent.

The relative activity of MnP was plotted against the hydrophobicity (C_o/C_w) of the substrates (Fig. 3). The hydrophobicity of the substrates was estimated using partition coefficients of the substrate for water and *n*-octanol. A positive relationship between both factors was found in the three groups of substrates, namely, anisidines, phenlenediamines and aminophenols.

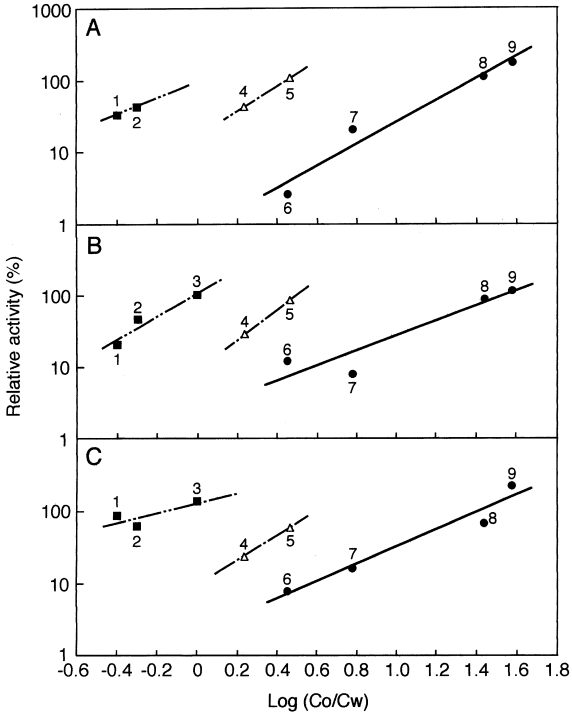

Fig. 3. Correlation between hydrophobicity of aromatic amines and MnP activity in aqueous 70% (v/v) acetone (A), acetonitrile (B) and diethylene glycol (C) media. The values of hydrophobicity of *m*-(1), *p*-(2) and *o*-phenylenediamines (3), *m*-(4) and *o*-aminophenols (5), *p*-(6), *o*-(7) and *m*-anisidines (8), and 3,3′-dimethoxybenzidine (9) were estimated as the partition coefficient of the substrates between water and *n*-octanol. These values were taken from Ref. [25].

4. Discussion

Manganese(II) peroxidase (MnP) has been isolated from the lignin-degrading basidiomycetes and the mechanism of the reaction catalyzed by this enzyme has been well studied. This enzyme catalyzes a less selective oxidation of various phenolic compounds and non-phenolic compounds in the presence of the appropriate intermediate [11,36]. MnP oxidizes Mn(II) to give Mn(III) which causes oxidation of aromatic compounds. The basic reaction was a one-electron oxidation of the substrates to produce phenoxy radicals using iron protoporphyrin IX as a cofactor. MnP is also characterized by a more positive oxidation-reduction potential (Em7) than other non-ligninolytic peroxidases [37]. These properties may enable a less selective oxidation of the substrates. This catalytic activity of MnP can be applied to the conversion and degradation of aromatic compounds. For the industrial use of this enzyme, a reaction in organic solvents would be advantageous, because the substrate aromatic compounds are insoluble or less soluble in water.

This research was carried out to examine the possibility of using MnP in organic solvents and to find the parameters which affect the reactivity of the substrates in organic solvents. To facilitate selection of the best solvents to optimize activity, the relationship between the activity of MnP and physicochemical parameters of organic solvents is thought to be important.

At first in this study, the activities of MnP were measured using various aromatic compounds in aqueous organic media. It was found that MnP retained its activity in several 70% (v/v) aqueous solutions of water-miscible organic solvents such as ethylene glycol, diethylene glycol, acetone and acetonitrile (Table 1). The conformation of MnP will be influenced by the solvent in which the enzyme was dissolved. As shown in Table 2, the absorption peak corresponding to the Soret band which appears at around 407 nm in UV/VIS absorption spectra of native MnP changed in the solvent which caused the loss of the activity.

The optimal pH of MnP in aqueous buffer was around pH 4.5. Addition of solvents was found to change the pH of the reaction mixture. Pyridine and chloroform in particular changed the pH of the reaction mixture resulting in the complete loss of the activity. However, it was not thought that change of the pH was the only the reason causing the loss of enzyme activity.

Organic solvents possibly affect each step of the catalytic cycle of MnP, namely, the oxidation of Mn(II) to Mn(III) and oxidation of substrate by Mn(III). Therefore, the effect of organic solvents on the oxidation of Mn(II) was examined. The Dimroth–Reichardt parameter $E_T(30)$ [38], which is an empirical parameter for the solvent polarity and is directly related to the free energy of the solvation process, has been reported to be representative of the changes in the three-dimensional structures due to solvent–protein interactions [39], and have been used for evaluating conformational changes of enzymes in the presence of organic solvents. As shown in Table 3 and Fig. 2, MnP showed higher activity to oxidize Mn(II) in the presence of solvents which have lower $E_T(30)$ values such as acetone, acetonitrile, DEGDE and 1,2-dimethoxyethane. These solvents were suggested to have little effect on the conformation of MnP protein based on the spectral analysis (Table 2). Therefore, it may be that Mn(II) ion, rather than MnP protein, is easily solvated by the molecules of organic solvents which have higher $E_T(30)$ values, and the solvated ion thus generated hindered the oxidation of Mn(II) to Mn(III) by MnP. In contrast to the results obtained in the present study, the activity of LiP was higher in the presence of organic solvents which have higher $E_T(30)$ values in a previous report [25]. Further works are required to obtain information about the effect of organic solvents on the conformation of MnP.

The oxidation of guaiacol by chemically prepared Mn(III) in aqueous organic media was investigated. The increase in absorbance due to the oxidation of guaiacol in 70% (v/v) aqueous acetone, acetonitrile, diethylene glycol, ethanol, ethylene glycol and methanol media was higher than that for the reactions in the other solvents (Table 4). These results showed that Mn(III) also catalyzes the oxidation of guaiacol in the aqueous organic media. It is worth noting that Mn(III) oxidized guaiacol in the presence of methanol, ethanol, ethylene glycol and diethylene glycol; activity for the oxidation of Mn(II) was absent or very low in the presence of these solvents. These results suggested that organic solvents affect the oxidation of Mn(II) by MnP rather than the oxidation of guaiacol by Mn(III).

Different types of aromatic compounds were oxidized by MnP in 70% (v/v) aqueous acetone, acetonitrile and diethylene glycol media (Table 5). The activity to oxidize 3,3′-dimethoxybenzidine in 70% (v/v) aqueous acetone, acetonitrile and diethylene glycol media was found to be stronger than that in the system without addition of the solvents. The MnP activity in these three aqueous water-miscible media was also greater than that of LiP from *P. chrysosporium* [24,25]. Therefore, it is concluded that MnP is more tolerant of organic

solvents than is LiP. Hydrophobic aromatic amines are suggested to be more reactive in aqueous acetone, acetonitrile and diethylene glycol media than hydrophilic aromatic amines (Fig. 3). Hydrophobic aromatic compounds have a higher binding energy necessary for catalytic activities than hydrophilic aromatics [40]. Therefore, less polar media will be preferable for the oxidation of aromatic substrates as shown for HRP [40] and LiP [25].

As mentioned above, various factors including the stability of the enzyme structure in organic solvents, solvation of the solvent with Mn(II) ion and protein and hydrophobicity of the substrates are involved in the reactivity of MnP in organic solvents. However, the complexity of the mechanism of the catalytic activity of MnP makes it difficult to analyze the factors influencing the reaction of MnP in organic solvents. Other parameters which control the reaction in organic solvents are now under investigation. The final goal of this research is to develop a reaction system which is applicable to the chemical process.

5. Conclusion

In this study, MnP from the lignin-degrading basidiomycete *B. adusta* was shown to oxidize various aromatic compounds in aqueous water-miscible organic media. MnP was also shown to oxidize directly Mn(II) in these media to give Mn(III) which further oxidizes aromatic compounds. MnP was found to be more tolerant than LiP in the organic solvents. The hydrophobicity of the substrates and the polarity of the solvents were found to be important factors influencing MnP activity in organic solvents.

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