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Role of Tween 80 in biobleaching of unbleached hardwood kraft pulp with manganese peroxidase

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Abstract The role of Tween 80 in biobleaching of unbleached hardwood kraft pulp (HWKP) with manganese peroxidase (MnP) was investigated. Among the surfactants (e.g., Tween 80, Tween 20, CHAPSO) the most significant brightness increase was obtained with Tween 80. Tween 80 and Tween 20 exhibited several effects, such as dispersion of degraded lignin and activation of MnP, that partly contributed to the brightening of HWKP during MnP treatment. However, these characteristics did not explain the most appreciable effect on the brightness increase by Tween 80. Lipid peroxidation of surfactants during MnP biobleaching was determined by measuring the peroxide value (POV). The order of the POV increase was consistent with that of the brightness increase of pulp during MnP treatment in the presence of various surfactants or linolenic acid. However, radicals and peroxides derived from lipid (linolenic acid) peroxidation by lipoxidase hardly brightened the HWKP by themselves. These results suggested that Tween 80 was peroxidized by Mn(III), and that Mn(III) and lipid peroxidation of Tween 80 synergistically brightened HWKP.

Key words Lignin · Manganese peroxidase · Biobleaching · Surfactant · Lipid peroxidation

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Introduction

In our previous paper we reported that in vitro manganese peroxidase (MnP) treatment could brighten unbleached hardwood kraft pulp (HWKP) and softwood kraft pulp.[1] The in vitro MnP system contains malonate buffer, $MnSO_4$ as Mn(II), Tween 80, and glucose oxidase (GOD) and glucose for supplying hydrogen peroxide. It is known that Tween 80 effectively improves the brightening of pulp by MnP biobleaching, but its role in biobleaching has not been clarified.

Tween 80 is a nonionic surfactant, and some nonionic surfactants are widely used for dispersing hydrophobic materials, such as polycyclic aromatic hydrocarbons (PAHs);[2–6] therefore, it may accelerate dispersion of the hydrophobic degraded lignin by MnP from the pulp into buffer solution during MnP treatment. On the other hand, Venkatadri and Irvine reported that surfactants, including Tween 80, protect lignin peroxidase (LiP) against mechanical inactivation due to agitation.[7] We previously showed that the polyethylene degradation by MnP in the absence of hydrogen peroxide requires surfactants such as Tween 80 to retain the MnP activity.[8,9] It is also reported that optimal concentration of anionic surfactant activates horseradish peroxidase.[10] According to these reports, it seems likely that Tween 80 positively influences the MnP activity during MnP biobleaching. Furthermore, it was reported that MnP promotes peroxidation of unsaturated fatty acid and that the MnP system containing unsaturated fatty acid accomplishes decomposition of nonphenolic lignin substructures[11,12] and phenanthrene,[13] which otherwise resists oxidation by MnP. Tween 80 contains unsaturated fatty acid (oleic acid), so the MnP/lipid peroxidation system may be involved in the brightening of HWKP during MnP treatment. In this study, to clarify the role of Tween 80 in MnP biobleaching, the effect of this surfactant on the brightening of HWKP and MnP activity were compared with those of some other surfactants, and involvement of the MnP/lipid peroxidation system in the brightening HWKP was also examined.

Materials and methods

Pulp, chemicals, and MnP

The HWKP (brightness 29.0%, kappa number 15.5) produced by Oji Paper Co. was used in this study. Tween 80 and Tween 20 of low peroxide and low carbonyl grade (Surfact-Amps) were provided by Pierce Chemical Co. All other chemicals were reagent grade. Partially purified MnP was prepared from the culture fluid of IZU-154 according to our previous report.[1]

Enzyme assay

The MnP activity was assayed by monitoring the oxidation of 2,6-dimethoxyphenol at 470nm. The reaction mixture contained 1mM 2,6-dimethoxyphenol, 50mM malonate buffer (pH 4.5), 0.1mM $MnSO_4$, and 0.2mM hydrogen peroxide. One unit of MnP activity is defined as the amount of enzyme releasing 1μmol of reaction product per minute.

Pulp treatment with MnP, lipoxidase, or Mn(III)

For MnP treatment of the HWKP, the pulp was suspended at a consistency of 1% in 50mM malonate buffer (pH 4.5) containing 75mU/ml partially purified MnP, 0.1mM $MnSO_4$, 25mM glucose, GOD 25mU/ml, and 0.05% (w/v) surfactant unless otherwise noted.

For lipoxidase treatment, HWKP was suspended at a consistency of 1% in 67mM borate buffer (pH 9.0) containing 3mM linolenic acid and lipoxidase (EC: 1.13.1.12) 130–500U/ml from soybean (type I-B, Sigma Chemical Co.).

For the Mn(III) treatment of HWKP, Mn(III) acetate was dissolved in 1M malonate buffer (pH 4.5) at a concentration of 200mM; then this high concentration solution and linolenic acid were added to the pulp suspended in distilled water. The final reaction mixture contained 1% HWKP, 50mM malonate buffer, 10mM Mn(III) acetate, and linolenic acid (1mM or 3mM).

These treatments were carried out at 37°C with stirring, and the pulp samples were then washed with water. Pulp sheets were prepared with a Buchner funnel (diameter 11mm) and air-dried. Brightness was determined with a colorimeter (model CR-300; Minolta). The values determined with the colorimeter were multiplied by a coefficient to adjust them to ISO brightness values.

POV assay

The peroxide value was assayed by the procedures of Takagi et al.[14] with some modification. A 0.2ml of freshly prepared 50% (w/v) aqueous KI solution was added to the mixture of the sample (0.9ml), chloroform (1ml), and acetic acid (2ml); and it was allowed to stand in the dark for 5min. The reaction was stopped by adding 0.9ml of 4.0% cadmium acetate dihydrate solution. After shaking, the solution was placed in the dark until the two phases were clearly separated. The absorbance at 410nm of the upper aqueous phase was measured spectrophotometrically.

Results

Comparison of surfactants on brightness increase

We performed the MnP treatment of HWKP in the presence or absence of surfactant. The pulp brightness increased by about 11 points in the absence of surfactant. On the other hand, the brightness increase was about 18.5 or 16.0 points in the presence of Tween 80 or Tween 20, respectively (Fig. 1). We also examined MnP treatment in the presence of other surfactants. All of these surfactants improved the brightening of HWKP, but none of them effectively brightened HWKP more than Tween 80 (Fig. 2). These results indicate that many surfactants improve the brightening of the pulp by MnP treatment, and that Tween 80 plays some specific, important roles that could not be found in other surfactants regarding the brightening of HWKP.

Dispersion of degraded lignin

The residual lignin in unbleached kraft pulp is regarded as hydrophobic material, which probably limits the brightness increase of the pulp during MnP treatment. Therefore, surfactants may accelerate dispersion of the degraded lignin from the pulp into buffer solution during the MnP treatment. To determine the dispersive effect of each surfactant, HWKP was treated with MnP for 6h or 24h in the absence of surfactant; then each of the treated HWKPs was washed with water, 0.05% Tween 80, or 0.05% Tween 20. The wash-

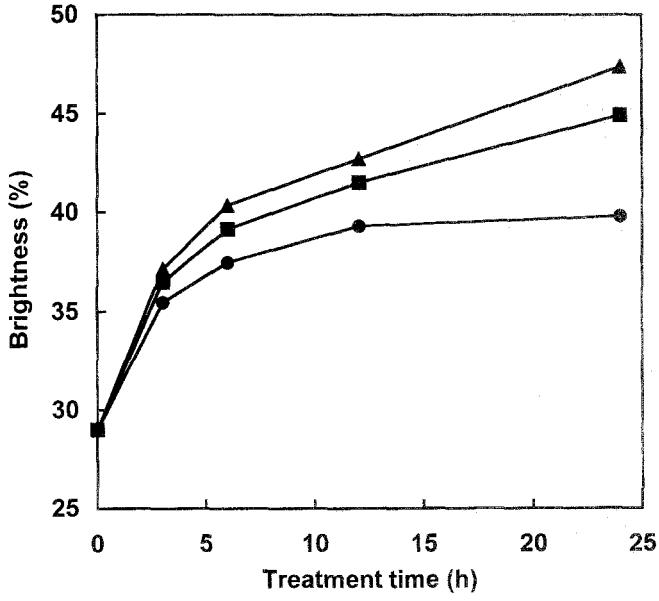

Fig. 1. Effect of surfactant on the brightness during MnP biobleaching. *Circles*, no surfactant; *squares*, Tween 20; *triangles*, Tween 80

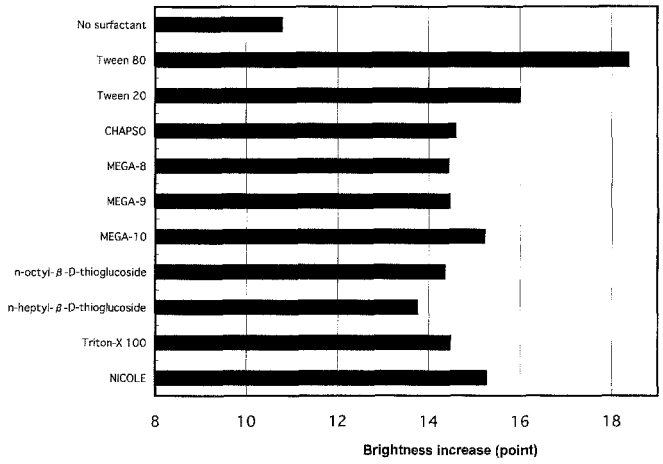

Fig. 2. Effect of surfactant on the brightness after MnP biobleaching for 24 h

Table 1. Washing with surfactant: effect on the brightness of MnP-treated hardwood kraft pulp

Wash solution	Brightness increase (point)	
	6 h[a]	24 h[b]
Water	0.07	0.06
Tween 80 0.05%	1.09	2.53
Tween 20 0.05%	1.24	2.63

[a] Washing for 6 h after the 6-h MnP treatment
[b] Washing for 24 h after the 24-h MnP treatment

ing time was synchronized with the MnP treatment time. The pulp brightness was increased by approximately 1.0 or 2.5 points by washing with surfactants for 6 h or 24 h, respectively, but no difference was observed in the brightness increase by Tween 80 and Tween 20. The increase in brightness was negligible with water washing (Table 1). This result indicates that the dispersion of the degraded lignin is improved by surfactants. As the surfactant is co-localized in the MnP biobleaching system, we speculated that the addition of surfactants may accelerate the contact between residual lignin in HWKP and active species [mainly Mn(III)]. This effect may also contribute to the brightening of HWKP during MnP biobleaching, but Tween 80 does not have any specifically higher dispersion effect.

Stabilization and activation of MnP

In the previous study on polyethylene degradation by MnP, it was found that the degradation of polyethylene membrane is accelerated by the addition of Tween 80 in the absence of hydrogen peroxide[8] and that Tween 80 acts as a stabilizer for MnP.[9] It was also reported that Tween 80 protects LiP against the mechanical inactivation due to agitation.[7] Therefore, we measured the residual MnP activity during MnP treatment.

In the absence of surfactant, the initial MnP activity was determined to be 75 mU/ml, which corresponded to the

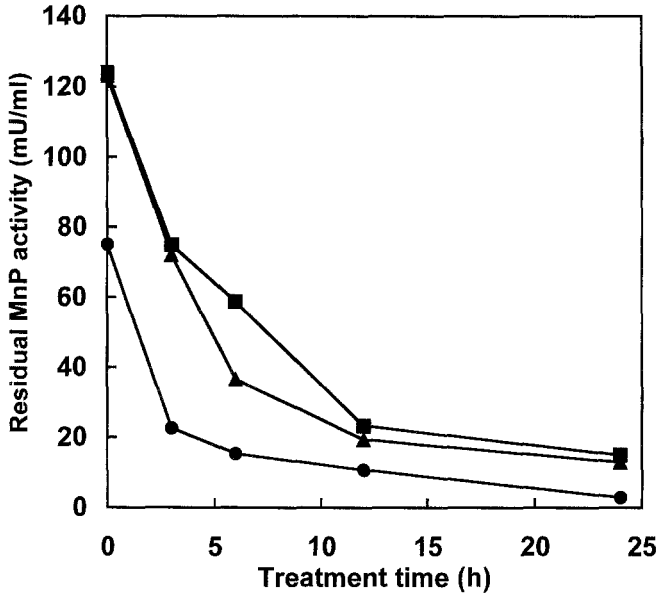

Fig. 3. Effect of surfactant on the residual enzyme activity during MnP biobleaching. See Fig. 1 for explanation of symbols

amount of MnP loaded. On the other hand, in the presence of surfactants the initial MnP activity was 120 mU/ml, that is, about 1.6-fold of the loaded MnP. Despite the fact that MnP activity decreased similarly with each MnP treatment, the residual MnP activities in the presence of surfactants were always higher than those in the absence of surfactant, because initial MnP activities were increased by surfactants (Fig. 3). This may contribute to the greater brightening of HWKP. However, the larger brightening effect of Tween 80 over that of Tween 20 could not be explained by stabilization and activation of MnP.

Lipid peroxidation of surfactant and linolenic acid

Tween 80 contains oleic acid in its molecule and was employed in MnP-dependent lipid peroxidation system that could depolymerize nonphenolic lignin substructures.[11] It is assumed that nonphenolic lignin in HWKP may be effectively degraded by MnP-dependent Tween 80 peroxidation. To clarify the involvement of peroxidation of Tween 80 to pulp brightening, the lipid peroxidation of surfactant (Tween 80, Tween 20, or CHAPSO) during MnP treatment was determined by measuring the peroxide value (POV). Tween 20 is composed of lauric acid (saturated fatty acid), and CHAPSO is not composed of any fatty acid. The POV for MnP treatment with CHAPSO was the same as that for the control (no surfactant), and the highest POV was observed with Tween 80. The order of the POV increase was consistent with that of the brightness increase (Fig. 4). To further investigate the effect of the lipid peroxidation on pulp brightening, Tween 80 was substituted by linolenic acid, and the pulp brightness and POV during the MnP treatment were determined. Brightness and POV synchronously increased with the concentration of linolenic acid

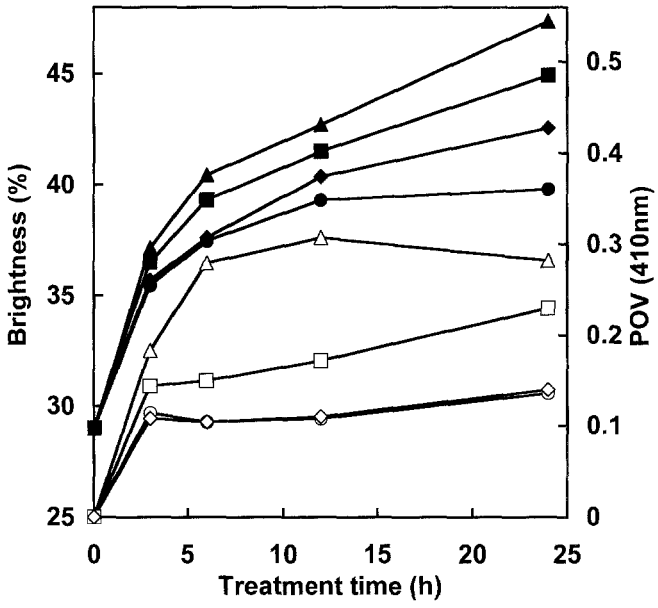

Fig. 4. Effect of surfactant on the brightness and peroxide volume (POV) during MnP biobleaching. *Circles*, no surfactant; *diamonds*, CHAPSO; *squares*, Tween 20; *triangles*, Tween 80. *Filled symbols* are for brightness; *open symbols* are for POV

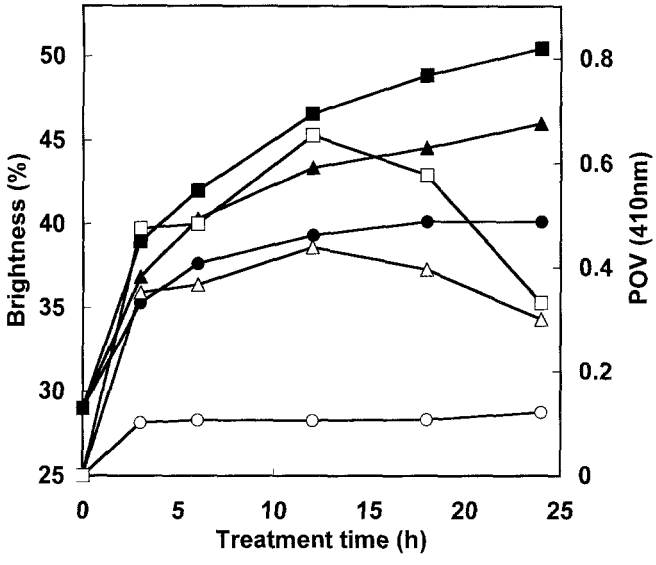

Fig. 5. Effect of linolenic acid on the brightness and POV during MnP biobleaching. *Circles*, 0 mM; *triangles*, 1 mM; *squares*, 3 mM. *Filled symbols* are for brightness; *open symbols* are for POV

(Fig. 5). It is thus obvious that Tween 80 is peroxidized during MnP biobleaching of HWKP and that the peroxidation strongly relates to the greatest brightening effect of Tween 80 among surfactants.

Decomposition of nonphenolic lignin substructures in the hydrogen peroxide-free MnP-dependent lipid peroxidation system[11–13] was previously reported, but the reaction mechanism is still unclear. It is of interest how the MnP/lipid peroxidation system is involved in the degradation of residual lignin in HWKP. To investigate the involvement of

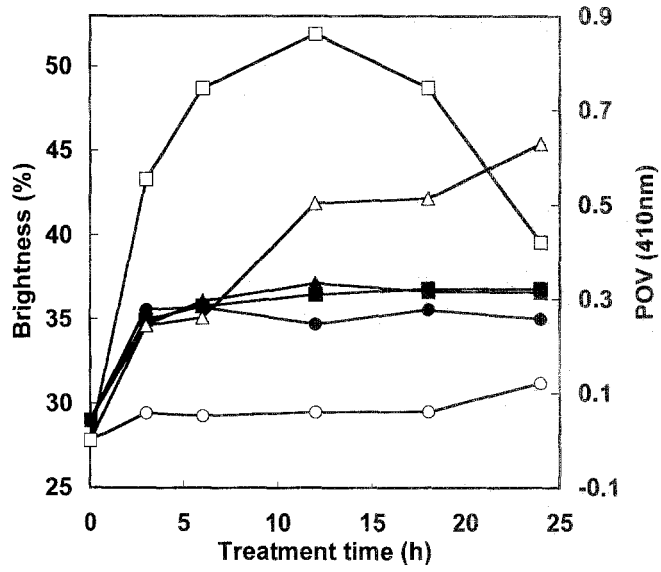

Fig. 6. Effect of lipid peroxidation on the brightness and POV during lipoxidase treatment. Lipoxidase: *circles*, 0 U/ml; *triangles*, 130 U/ml; *squares*, 500 U/ml. *Filled symbols* are for brightness; *open symbols* are for POV

Table 2. Changes in the brightness and POV by Mn(III) treatment

Condition	Brightness (%)	POV (410nm)
Control[a]	33.0	0.105
Mn(III) 0 mM, linolenic acid 3 mM	33.0	0.136
Mn(III) 10 mM	34.6	0.124
Mn(III) 10 mM, linolenic acid 1 mM	36.5	0.275
Mn(III) 10 mM, linolenic acid 3 mM	38.3	0.639

POV, peroxide value
[a] Control: Mn(III) and linolenic acid were omitted

peroxides and radicals derived from lipid peroxidation in the brightening of pulps, HWKP was subjected to the lipoxidase treatment in the presence of linolenic acid, and the pulp brightness and POV were monitored during the treatment. POV increased with the dose of lipoxidase, but the brightness of the pulp did not (Fig. 6). This result indicates that peroxides and radicals derived from lipoxidase treatment cannot brighten the pulp by themselves. Furthermore, generation of Mn(III) and an increase in brightness were not observed after addition of Mn(II) and malonic acid to the lipoxidase treatment (data not shown). This result also suggests that peroxides and radicals derived from lipoxidase treatment could not oxidize Mn(II) to Mn(III). From these observations it was inferred that generation of Mn(III) and lipid peroxidation are necessary for the larger brightening effect. Therefore, HWKP was treated with Mn(III) in the presence or absence of linolenic acid for 6 h. After Mn(III) treatment in the presence of 1 mM or 3 mM linolenic acid, the pulp brightness increased by 3.5 and 5.3 points, respectively. When the linolenic acid was omitted from the system, the brightness increase was 1.6 points (Table 2). This experiment shows that HWKP was

synergistically brightened by Mn(III) and peroxidation of linolenic acid.

Discussion

As shown in Fig. 3, the initial MnP activity in the presence of Tween 80 (containing unsaturated fatty acid) or Tween 20 (containing saturated fatty acid) were similarly increased by approximately 1.6-fold over the control (no surfactant). The same level of the increase in MnP activity was observed in the presence of other surfactants such as CHAPSO and Triton X-100 (data not shown). CHAPSO and Triton X-100 are not composed of any fatty acid; therefore, the increase in MnP activity induced by Tween 80 cannot be explained by peroxidation of unsaturated fatty acid. These effects of the surfactant may be due to a change in the enzyme conformation accompanied by the enhanced accessibility of enzyme-active sites to the substrate, as accounted for by activation of horseradish peroxidase (HRP) by anionic surfactant.[10]

Tween 80 and Tween 20 did not act as stabilizers for MnP (Fig. 3). This result conflicted with that in polyethylene degradation[9] in which initially added MnP retained its activity at least 4 days in the presence of these surfactants. It is well known that heme is denatured by the excess hydrogen peroxide.[15,16] More recently, it was reported that hydrogen peroxide is a major factor in inactivation of peroxidase in liquid culture of the ligninolytic fungus *Pleurotus pulmonarius*.[17] Hydrogen peroxide was supplied to the MnP biobleaching system by glucose and GOD but not to the polyethylene-degrading one. The faster inactivation of MnP during MnP biobleaching suggests that neither Tween 80 nor Tween 20 can rescue MnP from inactivation by hydrogen peroxide.[18]

Lipid peroxidation is a process that generates organic peroxide and organic peroxy and alkoxy radicals; and it is known to be induced by lipoxidase, hydroxy radical, and transition metal.[19,20] Dix et al. reported that heme can catalyze the oxidation of 7,8-dihydroxy-7,8-dihydrobenzo [*a*]pyrene by polyunsaturated fatty acid hydroperoxide.[21,22] Watanabe et al. also reported copper-dependent depolymerization of lignin in the presence of pyridine and organic hydroperoxide. This system could not depolymerize lignin in the absence of Cu(II).[23] These reports suggest the possibility that heme or transition metal(s) is fundamental to lignin degradation in the lipid peroxidation system, generating organic peroxides, radicals, or both. In our experiments, the brightness of the pulp did not increase after peroxidation of linolenic acid with lipoxidase in the presence of Mn(II). Furthermore, a brightness increase was also not observed in the following two experiments; HWKP was treated by HRP or *Arthromyces ramosus* peroxidase instead of MnP in the presence of linolenic acid. A hydrogen peroxide supplement was omitted during MnP biobleaching containing linoleic acid. These results indicate that the reaction system containing organic peroxide and heme or organic peroxide and Mn(II) does not contribute to degrading the residual lignin in HWKP, and that generation of Mn(III) is essential for pulp brightening in the presence of unsaturated fatty acid. Therefore, we checked the 2,6-dimethoxyphenol oxidation activity in the MnP treatment solution to determine the production of Mn(III). No difference was observed between the presence and absence of linolenic acid (data not shown). In the MnP biobleaching system containing GOD and glucose, the following two reactions are unlikely to be responsible for brightening the pulp: (1) Peroxide derived from the MnP/lipid peroxidation system is used by MnP as the electron acceptor as well as hydrogen peroxide; and (2) Mn(II) was oxidized to Mn(III) by peroxides or radicals (or both) derived from the lipid peroxidation.

The obviously higher POV and brightness increase in the presence of Mn(III) and linolenic acid than that in the absence of the latter are shown in Table 2. This result suggests that active agents, except Mn(III), are generated by the action of Mn(III) and unsaturated fatty acid, and that Mn(III) and active agents synergistically degrade the residual lignin in HWKP. In reported MnP-dependent lipid peroxidation systems without supplements of hydrogen peroxide,[11–13] the participation of Mn(III) in the decomposition of nonphenolic lignin substructures is not clear. However, recently Hofrichter et al. reported the oxidative decomposition of malonic acid as a basis for the action of MnP in the absence of hydrogen peroxide.[24] They proposed that the MnP catalytic cycle can be initiated by hydroperoxide and the hydrogen peroxide formed by the decomposition of malonate and the dismutation of superoxide anion radicals with Mn(II), respectively. Therefore, it is conceivable that Mn(III) is generated in the MnP-dependent lipid peroxidation system. In this system nonphenolic lignin may be degraded by a combination of Mn(III) and unsaturated fatty acid.

Details of the reaction(s) responsible for the brightness increase in the MnP biobleaching system containing unsaturated fatty acid are not available. At this point, we postulate that the peroxidation of unsaturated fatty acids such as Tween 80 or linolenic acid are accelerated by Mn(III), and that Mn(III) and the peroxidation of unsaturated fatty acids synergistically brighten HWKP during MnP biobleaching.

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