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Katsunobu Ehara · Yuji Tsutsumi · Tomoaki Nishida

Structural changes of residual lignin in softwood kraft pulp treated with manganese peroxidase

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Abstract Structural changes of residual lignin in unbleached softwood kraft pulp (SWKP) during manganese peroxidase (MnP) treatment were investigated to obtain some understanding of the biobleaching action of SWKP with MnP treatment. Alkaline-extracted lignin from darkened SWKP by MnP showed more intense color and contained more *o*-quinone than that from control SWKP. However, no difference in the conjugated α-carbonyl was observed between the lignins from MnP-treated and control SWKP. The nitrobenzene oxidation analysis revealed that oxidative condensation of non-condensed lignin in SWKP occurs during an early stage of MnP treatment. These observations were supported by the model experiment in which the lignin prepared from control SWKP was subjected to MnP treatments three times, and the changes of color and functional groups in the lignin were determined after each treatment. These results suggested that an increase in *o*-quinone and the condensation reaction of non-condensed lignin in SWKP are responsible for the characteristic darkening of SWKP during MnP treatment. It was also ascertained that darkened lignin was degraded and brightened by repeated MnP treatments.

Key words Kraft pulp · Residual lignin · Manganese peroxidase · Functional group · Alkaline nitrobenzene oxidation

Introduction

In a previous paper we reported that manganese peroxidase (MnP) can degrade residual lignins in unbleached hardwood kraft pulp (HWKP) and softwood kraft pulp (SWKP). No difference in the delignification rate by MnP treatment was observed between SWKP and HWKP. Whereas the brightness of HWKP increased with delignification, the brightness of SWKP decreased during the first period of MnP treatment and started to increase thereafter.[1] This finding indicates that some characteristic reactions responsible for the darkening of SWKP occur between the residual lignin and MnP.

A decrease in brightness during biobleaching using white-rot fungi[2–4] or ligninolytic enzymes[5,6] has also been reported. In those reports the brightness decrease during biobleaching was hypothetically explained by the formation of conjugated carbonyls and quinones via oxidation of residual lignin. However, quantitative data on the increase in these functional groups have not yet been shown, and the reason for the darkening of SWKP during fungal or MnP treatment remains unclear. Moreover, the increase in brightness of SWKP after transient darkening during fungal or ligninolytic enzyme treatments has not been well documented in the literature. Our previous data on alkaline nitrobenzene oxidation from the pulps during MnP treatment indicated that SWKP contained much more non-condensed type lignin than HWKP and that non-condensed lignin decreased markedly along with the decrease in pulp brightness during MnP treatment.[1] These results suggest that the oxidation and condensation reactions of non-condensed lignin by MnP is responsible for the brightness decrease of SWKP.

The purpose of this study was to investigate the characteristic mechanisms of the darkening and following brightening of SWKP by MnP treatment. Therefore, the structural changes of the residual lignin in SWKP during MnP treatment were traced by determining the functional groups and alkaline nitrobenzene oxidation analysis.

Materials and methods

Pulp and preparation of MnP

The SWKP (kappa number 28.4, brightness 30.0%) provided by Abekawa Paper was used. MnP was prepared from the culture of lignin-degrading fungus IZU-154 and

K. Ehara · Y. Tsutsumi · T. Nishida (✉)
Department of Forest Resources Science, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan

was partially purified according to our previous report.[1] MnP activity was assayed by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 415nm; 1 unit of MnP activity is defined as the amount of enzyme that increases the absorbance by 0.1/min.[1]

Preparation and fractionation of alkaline-extracted lignin from MnP-treated SWKP

The SWKP was treated with 100 units of MnP for 3h, according to our previous report.[1] Alkaline extractions of control SWKP and MnP-treated SWKP were performed at 60°C and 10% pulp consistency for 1h with a charge of 3.5% NaOH on pulp. After alkaline extraction, the alkaline-extracted solution was obtained by filtration. The solutions were acidified to pH 2.5 with dilute hydrochloric acid and then extracted with chloroform three times. After chloroform extraction, the residue was separated into a water layer and a precipitate by means of centrifugation (9000rpm, 10min). The precipitate was washed three times with water, freeze-dried, and then dissolved in dioxane/water (9:1). The alkaline extraction of control SWKP without MnP treatment and following fractionation were performed in the same manner (control experiments). Absorbances at 280, 400, and 470nm for all fractions were measured by an ultraviolet-visible spectrophotometer (Shimazu UV-160), and the absorbance ratios (400nm/280nm or 470nm/280nm) were determined.

Hypochromic effect by sodium borohydride or trimethyl phosphite treatment

The precipitate (1.5mg) from control SWKP or MnP-treated SWKP was dissolved in 3ml of dioxane/water (9:1) containing 0.01N sodium hydroxide or dry 2-methoxymethanol for the treatment with sodium borohydride or trimethyl phosphite, respectively. To each 3ml of the solution, 1.0mg of sodium borohydride or 93μl of trimethyl phosphite was added, and the reaction was lift for 45h at room temperature.

Analytical methods

Determination of carbohydrate in precipitate from alkaline-extracted solution was conducted according to the phenol sulfuric acid method of Fukui.[7] Two independent calibration curves using glucose and a dehydrogenative polymer of coniferyl alcohol (DHP) were prepared to estimate carbohydrate contents; the lignin content in the precipitate was then corrected by subtracting the carbohydrate content.

Alkaline nitrobenzene oxidation followed by gas chromatographic analysis was carried out according to the described methods.[8] The yields of the oxidation products, vanillin, vanillic acid, syringaldehyde, and syringic acid, were determined.

Conjugated α-carbonyl groups of precipitates were determined according to the method of Alder and Marton except that precipitates were dissolved in dioxane/water (9:1).[9] The amount of *o*-quinone of precipitates was calculated using the following formula by the absorbance decrease after the trimethyl phosphite treatment.[10]

$$o\text{-Quinone}\,(\text{mol/C}_9\ \text{unit}) = \Delta\varepsilon_a/\varepsilon = \left[\Delta A\ (C_9 - \text{UW})/bc\right]/\varepsilon$$

where ΔA = absorbance decrease due to trimethyl phosphite treatment; (C_9-UW) = molecular mass of lignin (190); b = cell length (1cm); c = concentration of lignin (g/l); and ε = Absorptivity of *o*-quinone of model compounds; 3,5-*di-tert*-butyl-1,2-benzoquinone (ε_{400} = 1931l/mol·cm), 5-ethyl-3-methoxy-1,2-benzoquinone (ε_{470} = 1450l/mol·cm).

Repeated MnP treatments of precipitate from control SWKP

The precipitate (0.1g) from control SWKP was dissolved in 2ml of dioxane/water (9:1). This solution was slowly added to 250ml of reaction solution containing 50mM malonate buffer (pH 4.5), partially purified MnP (250 units), 0.1mM $MnSO_4$, 25mM glucose, 2.5 units glucose oxidase (Wako Chemicals), and 0.05% Tween 80. The reaction was carried out at 37°C for 3h with stirring. After the MnP treatment, the reaction mixture was fractionated into a chloroform-soluble layer, water-soluble layer, and precipitate according to the above method. After the fractionation, the precipitate was repeatedly treated with MnP.

Results and discussion

Structural difference of alkaline-extracted lignin between control SWKP and MnP-treated SWKP

The brightness of SWKP was decreased from 30.0% ISO to 24.9% ISO by MnP treatment for 3h. However, the brightness of MnP-treated SWKP regained its initial level, and the kappa number of MnP-treated pulp decreased from 25.6 to 23.9 after the alkaline extraction (Table 1). It is clear that most of the colored lignin formed by MnP treatment, which corresponded to about 6.6% of residual lignin in MnP-treated SWKP, was extracted into the alkaline solution. Alkaline-extracted lignins from MnP-treated SWKP and control SWKP were further fractionated to obtain the most colored fraction. As shown in Table 2, the absorbance ratio of each fraction of the alkaline-extracted solution was generally higher in the MnP-treated SWKP than in the control SWKP. The most-colored lignin (higher absorbance ratio) was observed in the precipitate from MnP-treated SWKP.

Reid and Paice[3] and Paice et al.[6] explained that the brightness decrease of SWKP during treatment with fungal

Table 1. Changes in brightness and kappa number of SWKP by alkaline extraction

SWKP	Before alkaline extraction		After alkaline extraction	
	Brightness (%)	Kappa no.	Brightness (%)	Kappa no.
Control	30.0	28.4	30.8	26.3
MnP-treated	24.9	25.6	29.6	23.9

SWKP, softwood kraft pulp; MnP, manganese peroxidase.

Table 2. Total absorbance and absorbance ratio of each fraction of alkaline-extracted solution from control SWKP and MnP-treated SWKP

Fraction	Total absorbance[a]			Absorbance ratio	
	280 nm	400 nm	470 nm	400/280 nm	470/280 nm
Control					
All[b]	984.2	57.3	22.1	0.058	0.022
Chloroform	191.0	9.8	5.1	0.051	0.027
Water	397.8	26.4	10.4	0.066	0.026
Precipitate	285.3	14.6	5.8	0.051	0.020
MnP-treated					
All[b]	788.9	74.2	25.7	0.094	0.033
Chloroform	85.3	5.6	2.1	0.066	0.025
Water	413.2	30.4	10.8	0.074	0.026
Precipitate	218.2	27.1	9.3	0.124	0.043

[a] The products of absorbance and total volume (absorbance × milliliters).
[b] Alkaline-extracted lignin.

and ligninolytic enzyme may be due to the formation of quinones and conjugated carbonyls in residual lignin, although a quantitative analysis of these functional groups has not been done. It is well known that quinoids and conjugated carbonyls comprise the chromophore in lignin, and reduction of these functional groups by sodium borohydride results in less-colored lignin. It is also reported that *o*-quinone can be specifically converted to colorless oxyphosphorane adduct by trimethyl phosphite treatment.[10] Thus the most-colored fraction (precipitate) was subjected to these treatments, and changes in absorbance were determined (Table 3). The sodium borohydride treatment almost completely erased the absorbance increase by MnP treatment. The trimethyl phosphite treatment also erased the color of lignin caused by MnP treatment. The calculated contribution of *o*-quinone structure to the color of MnP-treated lignin was about 35%–38%.

Table 3. Hypochromic treatment of precipitates from control SWKP and MnP-treated SWKP

Wavelength (nm)	Absorbance and contribution[a] before and after treatment		
	Before	After $NaBH_4$	After $(CH_3O)_3P$
Control SWKP			
400	1.063	0.517	0.943
470	0.423	0.165	0.325
MnP-treated SWKP			
400	1.341	0.487 (110.8%)[a]	1.123 (35.3%)[a]
470	0.565	0.172 (95.1%)[a]	0.413 (38.0%)[a]

[a] The contribution was calculated by the formula:
Contribution (%) = $[(k - k_b)/k] \times 100$
where k is the absorbance difference between the precipitate of MnP-treated SWKP and that of control SWKP before hypochromic treatment, and k_b is the absorbance difference between the precipitate of MnP-treated SWKP and that of control SWKP after hypochromic treatment.

Determination of conjugated α-carbonyl and *o*-quinone and the alkaline nitrobenzene oxidation analysis were carried out for the precipitates (Table 4). The *o*-quinone in the precipitate from MnP-treated SWKP was much higher than that from the control SWKP. However, the conjugated α-carbonyls in the precipitates from MnP-treated SWKP and control SWKP were almost the same. This result was unexpected because the main degradation products from lignin model dimer or DHP with MnP were of an α-carbonyl structure,[11–13] and we showed that sodium borohydride treatment of the precipitate completely erases the color in lignin caused by MnP treatment (Table 3). In the case of alkaline nitrobenzene oxidation analysis, the yield of oxidation products in the precipitate from MnP-treated SWKP was lower than that from control SWKP. The decreased yield of oxidation products suggested a condensation reaction of non-condensed lignin. From these results, darkening of residual lignin in SWKP by MnP treatment may be explained by the following reactions: The increase in *o*-quinone resulted from the oxidative demethylation of lignin by MnP and the condensation of lignin, which led to the formation of a long conjugated structure.

Repeated MnP treatments of precipitate from control SWKP

In our previous study we observed the brightness increase and kappa number decrease of SWKP by increasing the MnP treatment time or by repeated MnP treatments.[1] This finding indicates that the residual lignin including the chromophore in SWKP is degraded by MnP treatment after the transient darkening. To investigate the structural change of residual lignin responsible for the darkening and following brightening of SWKP by MnP treatment, we attempted a study in which the precipitated lignin prepared from control SWKP was repeatedly treated with MnP, and the changes in color and functional groups were investigated. The chloroform- and water-soluble lignins may be of lower molecular weight than the precipitated lignin and are considered to correspond to the lignins released from pulps into the reaction solution during the MnP treatment of SWKP.

As shown in Table 5, the first MnP treatment increased the absorbance ratio of the precipitated lignin prepared from control SWKP, which may be the reaction that causes darkening of residual lignin in SWKP by MnP treatment. The precipitate prepared after the first MnP treatment was subjected to another MnP treatment. The second MnP treatment effected little change in the color of the precipitated lignin. In contrast, the absorbance ratios of the precipitate and the solution were decreased after the third MnP treatment, which means that the structural changes of the precipitated lignin after this MnP treatment may be responsible for the brightening of SWKP by MnP treatment. In addition, the total absorbances at 280 nm of chloroform- and water-soluble lignins obtained from the third MnP treatment were much higher than those from other MnP treatments, which indicates that the lignin darkened by the first MnP treatment can be degraded by the repeated MnP treatments.

Changes in *o*-quinone, conjugated α-carbonyl, and the yield of alkaline nitrobenzene oxidation products through repeated MnP treatments are summarized in Table 6. The conjugated α-carbonyl in each precipitate remained at almost the same level throughout the repeated MnP treatments. This finding supports the results in which there was no increase in conjugated α-carbonyl in the precipitate from control and MnP-treated SWKP, although a higher absorbance ratio was observed in the later precipitate (Tables 2, 4). On the other hand, the changes in *o*-quinone correlates well with the color change of lignin; the absorbance ratio and *o*-quinone increased during the first MnP treatment and decreased during the third MnP treatment. Neither showed significant changes with the second MnP treatment. Degra-

Table 4. Functional groups and alkaline nitrobenzene oxidation analyses of precipitates from alkaline-extracted solution

Precipitate	Conjugated α-carbonyl (mol/C_6–C_3)	*o*-Quinone (mol/C_6–C_3)		Yield of oxidation products (% on lignin)
		At 400 nm	At 470 nm	
Control SWKP	0.035	0.029	0.032	9.0
MnP-treated SWKP	0.039	0.053	0.050	5.4

Table 5. Repeated MnP treatments of precipitates from control SWKP

MnP treatment	Fraction	Total absorbance[a]			Absorbance ratio	
		280 nm	400 nm	470 nm	400/280 nm	470/280 nm
Before						
	Precipitate	1638.0	91.6	33.0	0.056	0.020
After the first treatment						
	MnP-treated solution	1586.0	229.4	66.9	0.145	0.042
	Chloroform	24.1	1.3	0.5	0.054	0.021
	Water	21.9	3.0	1.4	0.137	0.064
	Precipitate	1320.2	235.8	67.6	0.179	0.051
After the second treatment						
	MnP-treated solution	1283.4	206.0	60.3	0.161	0.047
	Chloroform	18.5	1.4	0.6	0.076	0.032
	Water	27.8	3.9	1.2	0.140	0.043
	Precipitate	1085.8	185.4	56.3	0.171	0.052
After the third treatment						
	MnP-treated solution	1003.9	120.6	39.5	0.120	0.039
	Chloroform	45.7	3.4	1.6	0.074	0.035
	Water	93.6	10.1	3.0	0.108	0.032
	Precipitate	742.2	107.2	32.0	0.144	0.043

[a] The products of absorbance and total volume (absorbance × milliliters).

Table 6. Structural changes of precipitates during repeated MnP treatments

Precipitate	Conjugated α-carbonyl (mol/C_6–C_3)	o-Quinone (mol/C_6–C_3)		Yield of oxidation products (% on lignin)
		400 nm	470 nm	
Before MnP treatment	0.035	0.029	0.032	9.0
After first MnP treatment	0.038	0.052	0.043	6.0
After second MnP treatment	0.038	0.048	0.043	5.3
After third MnP treatment	0.033	0.022	0.020	4.8

dation products of quinone structure were also observed in the in vitro degradation of lignin model dimer or DHP with MnP.[11–13] These results demonstrate that the formation and degradation of this structure in lignin by MnP treatment comprise one of the concerns with respect to the darkening and brightening of SWKP during MnP treatment.

The yield of alkaline nitrobenzene oxidation products markedly decreased after the first MnP treatment, but after the second MnP treatment the yield of oxidation products remained almost the same. This finding coincided with the results of our previous study: The yield and brightness of SWKP decreased during the first 3- or 6-h MnP treatment, and the yield of oxidation products remained the same after 6 h when the brightness increased gradually.[1] Furthermore, we previously reported that there was much guaiacyl type noncondensed lignin (which would be more susceptible to further polymerization or repolymerization by MnP[13]) in SWKP than in HWKP.[1] This difference may cause the difference in the brightening action between SWKP and HWKP during MnP treatment.

Conclusions

The structural changes of the residual lignin in swkp during MuP treatment were investigated. Oxidation of noncondensed lignin resulting in an increase in o-quinone and condensation reaction by MnP may lead to the darkening of lignin. The darkened lignin due to MnP treatment was degraded and brightened by extended or repeated MnP treatments.

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