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Biobleaching of Softwood Kraft Pulp by White-Rot Fungi and Its Related Enzymes*1

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白色腐朽菌による針葉樹クラフトパルプのバイオブリーチングとそれに関与する酵素*1

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Phanerochaete chrysosporium 及びカワラタケによる針葉樹クラフトパルプ (SWKP) のバイオブリーチングを行い，産生される酸化系酵素の役割を検討した。両菌株ともラッカーゼ (Lac) 及びマンガンペルオキシダーゼ (MnP) を産生したが，リグニンペルオキシダーゼの産生は認められなかった。カワラタケ処理による SWKP の脱リグニンは *P. chrysosporium* 処理と比べて少なかったが，Lac はより多量に産生されていたため SWKP の脱リグニンに及ぼす Lac の関与は小さいと判断された。一方，SWKP の *P. chrysosporium* 処理を広葉樹クラフトパルプ (HWKP) の場合と比較すると，脱リグニンは生じていたが，産生される MnP 量が著しく少なかった。また，Mn (II) 無添加系で部分精製 MnP により SWKP と HWKP を処理した結果，脱リグニンは HWKP のみで観察された。一方，SWKP に Mn(II) を添加すると，*P. chrysosporium* による MnP 産生と脱リグニンが促進され，MnP 処理による SWKP の脱リグニンも認められた。よって，十分量の Mn(II) が存在する条件下では MnP が SWKP の脱リグニンに関与しているものの，Mn (II) がほとんど存在しない条件下では MnP 以外の酵素が関与しているものと推察された。

The roles of oxidizing enzymes produced during the biobleaching of unbleached softwood kraft pulps (SWKPs) by *Phanerochaete chrysosporium* and *Trametes versicolor* were investigated. Both fungi produced laccase and manganese peroxidase (MnP), but lignin peroxidase production was not observed. While no appreciable delignification of SWKP was obtained by *T. versicolor*, laccase was produced extensively with this fungus compared with *P. chrysosporium* which showed greater delignification. The MnP production during the delignification of SWKP by *P. chrysosporium* was much less than that of unbleached hardwood kraft pulp (HWKP). Furthermore, *in vitro*, isolated MnP cannot degrade residual lignin in SWKP without the addition of Mn(II), although MnP can degrade that in HWKP. The addition of Mn(II) enhanced the MnP production and delignification during the biobleaching of SWKP by *P. chrysosporium*. The delignification of SWKP by an *in vitro* MnP treatment also was obtained with Mn(II)-addition. Therefore, MnP may be involved mostly in the delignification of SWKP under Mn(II)-sufficient conditions, although other enzymes, except MnP, may contribute to the delignification of SWKP under Mn(II)-insufficient conditions.

Keywords: biobleaching, softwood kraft pulp, white-rot fungi, lignin degradation, manganese peroxidase.

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1. INTRODUCTION

Growing public concern about the environment is giving rise to technological changes in pulp bleaching because the effluent from the conventional bleaching process contains numerous chlorinated organic compounds that have shown mutagenic activity.[1,2] There is a great interest, therefore, in eliminating or reducing the use of chlorine-based chemicals in bleaching, and at the same time, in developing alternative bleaching techniques which do not discharge hazardous compounds. From this viewpoint, much research has been done in an attempt to delignify and brighten unbleached kraft pulp by white-rot fungi which are the most effective lignin-degrading microorganisms.

Kirk and Yang were the first to recognize that unbleached softwood kraft pulp (SWKP) can be delignified partially by *Phanerochaete chrysosporium* Burds. when the fungal treatment is followed by alkaline extraction.[3] Furthermore, we showed that this fungus also can delignify and brighten unbleached hardwood kraft pulp (HWKP) in the solid-state fermentation system and increase its brightness by 22 points after two days of incubation.[4] On the other hand, Kirkpatrick *et al.* reported that *Trametes versicolor* (L.: Fr.) Pilat can decrease the residual lignin content substantially and increase the brightness of HWKP within three to five days,[5,6] and Reid *et al.* showed that this fungus does not delignify SWKP extensively without a subsequent hot-alkaline extraction and does not increase significantly its brightness within 14 days.[7] These results indicate that the responses of SWKP and HWKP to fungal bleaching are different, and the residual lignin in SWKP is more resistant to brightening and delignification by white-rot fungi.

In the series of previous studies, we investigated the roles of oxidizing enzymes (namely, manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase) and reducing enzymes in the biobleaching of HWKP by *P. chrysosporium* and *T. versicolor*. We showed that MnP plays the most important role in brightening and delignifying HWKP,[4,8] as shown in other reports by Kondo *et al.*[9] and by Harazono *et al.*[10] Here, to clarify the roles of oxidizing enzymes in the biobleaching of SWKP, we examined the relationship between the delignification of SWKP and the cumulative activities of oxidizing enzymes produced by white-rot fungi in the solid-state fermentation system with a low nitrogen-high carbon (LN-HC) culture medium in which Mn(II) either was added or not, and compared with that in the biobleaching of HWKP. *In vitro* treatments of SWKP and HWKP with partially purified MnP under conditions in which Mn(II) either was added or not to pulps, also were performed to study the difference in the delignification of SWKP and HWKP by MnP. From these results, we propose that other enzymes, except MnP, may contribute to the delignification of SWKP.

2. MATERIALS AND METHODS

2.1 Microorganisms and biobleaching of kraft pulp

Phanerochaete chrysosporium ME-446 and *Trametes* (*Coriolus*) *versicolor* IFO-30340 were used in this study. Fungal treatments of SWKPs (normal-lignin-content SWKP (SWKP-N): kappa no., 28.4, low-lignin-content SWKP (SWKP-L): kappa no., 18.8) and HWKP (kappa no., 15.5) in the solid-state fermentation system with an LN-HC culture medium was performed as described in our previous paper,[4] unless otherwise noted. To study the effects of the addition of Mn(II) on the biobleaching of SWKP-N by *P. chrysosporium*, 50 μg of Mn(II) per gram of pulp was added as $MnSO_4 \cdot H_2O$ to the fungal treatment.

2.2 Preparation and purification of MnP

Potato dextrose agar plates were inoculated with *P. chrysosporium* and incubated for five days at 30°C. Five disks punched from the grown edge of the mycelium were homogenized for 30s with 50 ml of PMY culture medium (3.0% glucose, 1.0% peptone, 1.0% malt extract, 0.4% yeast extract) and then put into a 500-ml Erlenmeyer flask with 150 ml of PMY culture medium and shaken at 200 rpm to give a mycelium suspension culture. After three days, 50 ml of the culture was homogenized once again, and then further precultured in a 500-ml Erlenmeyer flask with 250 ml of PMY culture medium on a rotary shaker (200 rpm) for three days at 30°C. The precultured mycelium of the fungus was separated from the PMY culture medium and then aseptically added to a 500-ml Erlenmeyer flask with 200 ml of LN-HC culture medium[4] containing 2.5 mM veratryl alcohol, 200 μM $MnSO_4$, and 0.05% Tween 80. The flasks were purged with oxygen every day and shaken at 200 rpm at 30°C.

After three days of incubation, mycelium was removed by centrifugation (10000 g) and the supernatant was concentrated by a factor of about 10 by ultrafiltration (ADVANTEC; 10000 - molecular-weight cut off). The enzyme concentrate was loaded onto a DEAE-TOYOPEARL (TOSOH) column equilibrated with 10 mM acetate buffer (pH 4.0). The column was eluted with the same buffer containing 100 mM NaCl. Fractions containing MnP activity were pooled and separated by gel permeation on a Superdex 75 HR 10/30 (Pharmacia Biotech) column with 10 mM acetate buffer (pH 4.5). MnP-enriched fractions further were subjected to a Mono Q (Pharmacia Biotech) column equilibrated with 10 mM acetate buffer (pH 4.5), then eluted with a linear gradient from 0 to 100 mM NaCl in the same buffer. Laccase and LiP activities were not detected in this partially purified MnP fraction.

2.3 MnP treatment of Pulp

For enzymatic treatments of pulps, 2 g of unbleached kraft pulp was suspended at a consistency of 1% in 200 ml of 50 mM malonate buffer (pH 4.5) containing partially purified MnP, 0.1 mM $MnSO_4$, 25 mM glucose, 5 U of glucose oxidase (Wako Chemicals), and 0.05% Tween 80.[9] The suspension was stirred at 37°C for 12 h. For the treatment without the addition of $MnSO_4$, modifications were made in the MnP treatment as follows: 2 mM oxalate and 50 mM succinate buffer (pH 4.5) were used.[10] For each control experiment, the pulp was treated in the same way except without the addition of MnP.

2.4 Enzyme assays

In the fungal treatments of pulps, MnP, LiP, and laccase activities were determined by the method described previously.[4] In the isolated MnP treatments of pulps, MnP activity was determined by the method of Paszcyñski *et al.*[11] One unit of activity of each enzyme is defined as the amount of enzyme that changes the absorbance by 0.1 per min, and the enzyme activity is expressed in units per gram of treated pulp in the case of fungal treatment. Data are means of duplicate analyses.

2.5 Pulp properties

After the fungal or enzymatic treatments, pulp samples were washed with water, and pulp sheets were prepared with a Buchner funnel (diameter, 11 mm) and air dried. Brightness and kappa number were determined by the method described previously.[4] The klason lignin content was determined according to JIS (Japan Industrial Standard) P8012-1962. Manganese ion contents in unbleached kraft pulps (UKPs) were determined by atomic absorption spectrophotometry.

3. RESULTS AND DISCUSSION

3.1 Biobleaching of SWKPs by white-rot fungi

The normal-lignin-content SWKP-N and low-lignin-content SWKP-L were incubated with *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with an LN-HC culture medium which was suitable for biobleaching of HWKP.[4] Figure 1 shows changes in the brightness and kappa number of SWKPs observed during treatments with these fungi for six days. The pulp brightness of both SWKPs decreased remarkably by the treatment with *T. versicolor*. No appreciable decrease in kappa number suggests that *T. versicolor* hardly degrades the residual lignin in both SWKPs during a six-day incubation period. In the case of the treatment with *P. chrysosporium*, the brightness of SWKP-N decreased during the first two days of incubation, then started to increase. However, the brightness of SWKP-N did not regain its initial level even after the fungal treatment of six days. In the biobleaching of SWKP-L by *P. chrysosporium* and *T. versicolor*, the brightness slightly increased on the first day of incubation, but it also decreased on the 2nd day of incubation. On the first day of incubation, a kappa number decrease was not observed; therefore, this brightening may not due to delignification by the fungi. The brightness of SWKP-L recovered the initial brightness after five days of incubation by only *P. chrysosporium* (Fig. 1). In our previous report, brightness of HWKP which had almost the same kappa number as SWKP-L steadily increased by the fungal treatment.[4] It is likely, therefore, that the difference in brightening performances between SWKP and HWKP by fungal treatments does not depend on the lignin content in pulps, but rather on the lignin structures in the pulps. Reid *et al.* also reported that *T. versicolor* increases the chromophore content of residual lignin in SWKP as well as solubilizing it during the first week of incubation, and proposed the possibility that the reactions which produce the chromophore such as

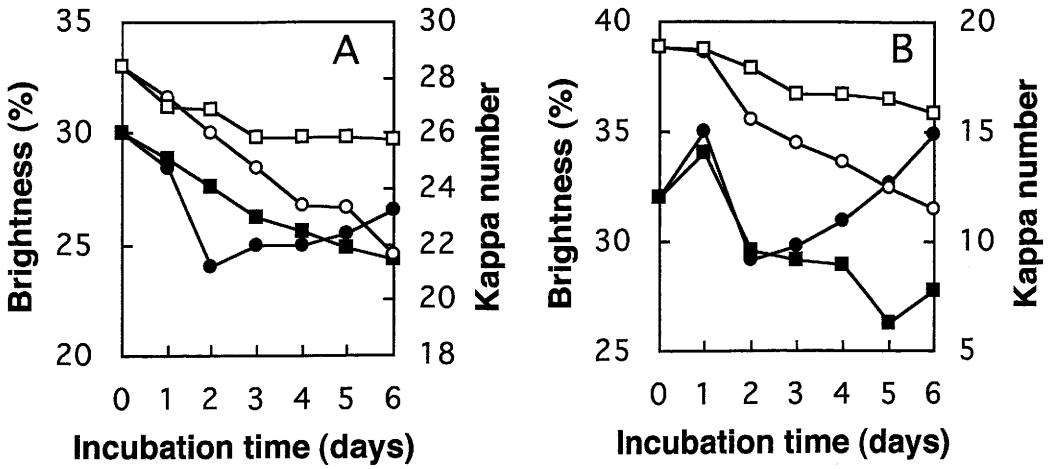

Fig. 1. Changes in the brightness and kappa number of SWKPs during treatment with *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with an LN-HC culture medium.
Legend: ●: *P. chrysosporium*, brightness; ■: *T. versicolor*, brightness; ○: *P. chrysosporium*, kappa number; □: *T. versicolor*, kappa number. (A) SWKP-N. (B) SWKP-L.

quinones and conjugated carbonyls are necessary steps in the degradation of lignin.[7] Therefore, the transient darkening and slow brightening of SWKP by fungal treatment seem to be characteristic and these phenomena cause difficulty in the biobleaching of SWKP by white-rot fungi. On the other hand, the kappa number of SWKPs decreased steadily from 28.4 to 21.6 for SWKP-N and from 18.8 to 11.5 for SWKP-L after six days of incubation with *P. chrysosporium* (Fig. 1). This result indicates that *P. chrysosporium* has a greater potential for delignification of SWKPs than does *T. versicolor* under the condition used in this study.

3.2 Enzymes related to delignification of SWKP by white-rot fungi

In the series of previous studies, we investigated the relationship between brightening of HWKP and the cumulative enzyme activity (total activity detected every 24 h during fungal treatment), and showed a linear relationship between the brightness increase of HWKP and the cumulative MnP activity in the solid-state fermentation system with both *P. chrysosporium* and *T. versicolor*. It also was confirmed that brightness increase is directly proportional to the degradation of residual lignin in HWKP.[4] In this experiment, however, brightness increase of SWKP was not proportional to its kappa number decrease during the treatment with *P. chrysosporium* and *T. versicolor*. Thus, the relationship between cumulative enzyme activities and delignification of SWKPs and HWKP during treatment with *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with an LN-HC culture medium was examined.

Laccase and MnP activities were detected during treatment of SWKP-N and SWKP-L with *P. chrysosporium* and *T. versicolor*. However, LiP activity was not detected throughout the treatment of SWKPs by both fungi, while we had shown previously that LiP activity was detected in the biobleaching of HWKP by *P. chrysosporium* and a linear relationship between cumulative LiP activity and delignification of HWKP was found.[4] Under the condition used here, *T. versicolor* hardly decreased the kappa number of SWKPs as shown in Fig. 1, but cumulative activity of laccase produced by *T. versicolor* was much more than that by *P. chrysosporium* which was shown to have greater ligninolytic activity (Table 1). This discordance implies that laccase may not be involved in the degradation of residual lignin in SWKP. In the treatment of HWKP with white-rot fungi, MnP is the most important enzyme.[4] Therefore, we investigated the relationships between cumulative MnP activities

Table 1. Changes in kappa number decrease, cumulative MnP activity and cumulative laccase activity during treatment of SWKP-N with *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with an LN-HC culture medium.

Incubation time (days)	*P. chrysosporium*			*T. versicolor*		
	Kappa number decrease (%)	Cumulative MnP activity (unit/g)	Cumulative laccase activity (unit/g)	Kappa number decrease (%)	Cumulative MnP activity (unit/g)	Cumulative laccase activity (unit/g)
2	10.0	36.2	36.3	2.5	80.6	61.5
4	18.7	84.3	63.4	4.7	97.9	270.2
6	22.8	118.3	90.8	6.5	110.7	502.1

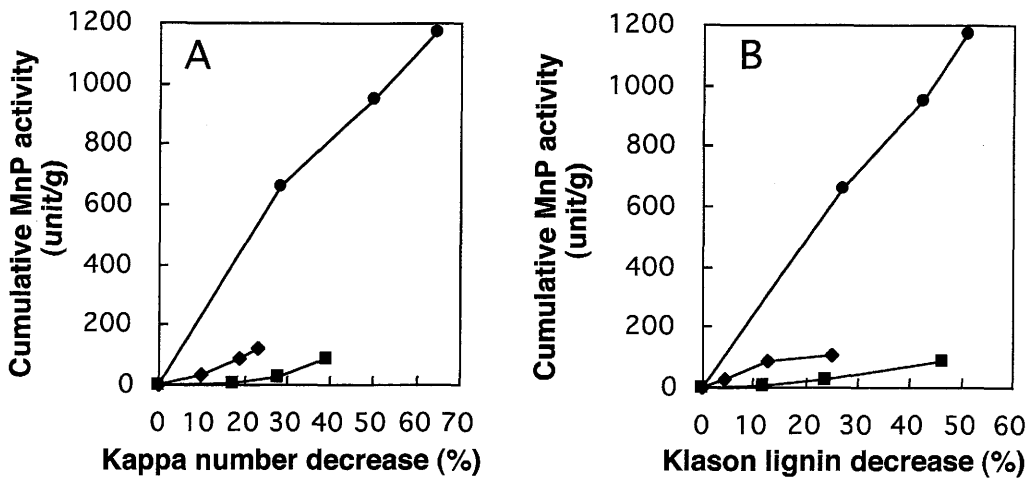

Fig. 2. Relationship between the cumulative MnP activity and delignification (A : kappa number decrease, B : klason lignin decrease) during treatment with *P. chrysosporium*.
Legend : ●: HWKP ; ◆: SWKP-N ; ■: SWKP-L.

and the kappa numbers or klason lignin decreases of SWKPs and HWKP during treatments with *P. chrysosporium*. As shown in Table 1, the cumulative activity of MnP produced for six days of incubation was about 100 unit/pulp (g) in the treatment of SWKP-N with *P. chrysosporium* and *T. versicolor*. This amount of activity corresponded to about one-tenth of the cumulative activities in the treatments of HWKP with both fungi. At the same level of kappa number decrease (Fig. 2A) or klason lignin decrease (Fig. 2B), cumulative MnP activities were much less for the SWKPs than for the HWKP. In other words, degradation of residual lignin was much greater in SWKPs than in HWKP at the same levels of cumulative MnP activities. Furthermore, although the kappa number decrease during treatment with *P. chrysosporium* was greater than that with *T. versicolor*, cumulative activities of MnP produced by *P. chrysosporium* and *T. versicolor* were almost at the same levels for four and six days of incubation (Table 1). From these results, it is questionable whether MnP relates to the delignification of SWKPs during treatments with both fungi, although we have shown that MnP is involved in the lignin degradation of HWKP.[4]

To clarify whether MnP produced during fungal treatment can degrade the residual lignin in SWKP, *in vitro* delignifications of SWKP and HWKP by partially purified MnP were compared under the conditions with and without the additions of Mn(II). Although Mn(II) is essential for the MnP action, Harazono *et al.* reported that *in vitro* MnP treatment of HWKP can be achieved without exogenous Mn(II) in the presence of oxalate.[10] The results listed in Table 2 show that MnP treatment decreased the kappa number of HWKP and increased its pulp brightness without the addition of Mn(II), which is in agreement with the result of Harazono *et al.*[10] In the case of SWKP-N treated with MnP under the condi-

Table 2. *In vitro* MnP treatments of UKP with and without additions of Mn(II).

Pulp	MnP treatment	Kappa number	Brightness (%)
HWKP	Control	14.0	34.3
	Mn(II) no addition	11.3	38.5
	Mn(II) addition	10.3	43.9
SWKP-N	Control	28.3	32.4
	Mn(II) no addition	28.3	32.4
	Mn(II) addition	21.9	31.6

tion without the addition of Mn(II), however, its kappa number was the same as that of the control pulp and no delignification was observed. On the other hand, the addition of Mn(II) initiated degradation of the residual lignin in SWKP-N by MnP, and the kappa number was decreased from 28.3 to 21.9. Such a different MnP action in the delignification of HWKP and SWKP-N under the condition without the addition of Mn(II) implies that SWKP-N does not contain enough manganese ion which is necessary for the MnP catalytic system.[12,13] This assumption was supported by the determination of the manganese ion contents in the pulp, which revealed that HWKP, SWKP-N, and SWKP-L contain 34 μg, 3 μg, and 5 μg of manganese ion per gram of pulp, respectively. Therefore, in the treatment of SWKP with the fungi, *P. chrysosporium* and *T. versicolor*, MnP produced during the fungal treatment may not participate in the degradation of the residual lignin in SWKPs. This idea explains the fact that *T. versicolor* showed lesser ligninolytic activity than did *P. chrysosporium* (Fig. 1), although both fungi produced the same levels of cumulative MnP activity during the treatment of SWKP (Table 1), and leads to the hypothesis that some other enzymes, except MnP, may be involved in the delignification of SWKP by *P. chrysosporium*. The production of the enzymes may be characteristic in the treatment with *P. chrysosporium* under a manganese ion deficient condition. Further investigation of the enzyme will be a subject in a forthcoming study.

3.3 Effect of Mn(II) addition on biobleaching of SWKP by P. chrysosporium

A lower MnP production by *P. chrysosporium* in the biobleaching of SWKPs may be due to the low content of manganese ions in SWKPs, because manganese ion is recognized to play an important role in the ligninolytic system, particularly as an inducer of MnP and a mediator of its catalytic cycle.[12-16] It is, therefore, interesting whether MnP would be effectively produced and delignification of SWKP would be accelerated in fungal treatment with the addition of Mn(II). Thus, treatment of SWKP-N with *P. chrysosporium* was done with the adding of 50 μg of Mn(II) as $MnSO_4$ per gram of pulp, and the cumulative MnP activity, kappa number decrease, and brightness increase were determined. MnP produc-

Table 3. Effects of Mn(II) addition on MnP production during biobleaching of SWKP-N by *P. chrysosporium*.

Incubation time (days)	Cumulative MnP activity (unit/g)	
	Addition of Mn (II) (μg/g of pulp)	
	0	50
2	25	491
4	89	608
6	110	684

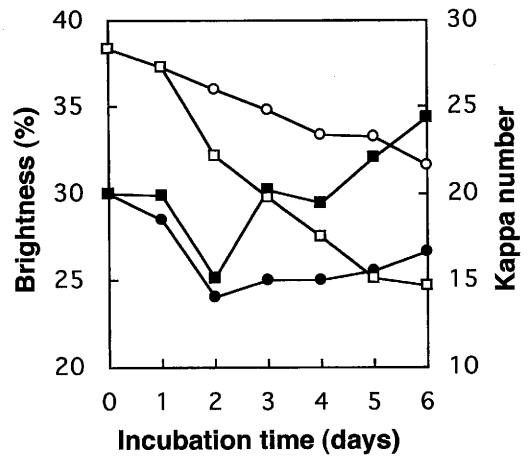

Fig. 3. Time courses of kappa number and brightness of SWKP-N during treatments with *P. chrysosporium* under conditions with and without the addition of Mn(II).
Legend: □: Mn(II) addition, kappa number; ■: Mn(II) addition, brightness; ○: control (no addition), kappa number; ●: control, brightness.

tion was accelerated by the adding of Mn(II) and the cumulative MnP activity after six-day incubation reached to about six-fold of the control without the addition of Mn(II) (Table 3). Simultaneously, the kappa number decrease and the brightness increase of SWKP-N became greater compared to those of the control by the addition of Mn(II) (Fig. 3). This is in agreement with the result of Kerem and Hadar that degradation of lignin in cotton branches and stalks by *Pleurotus ostreatus* (Jaeq. ex Fr.) Kumm. is enhanced by the addition of Mn(II) to the fermentation medium.[17,18] These results suggest that MnP induced by the addition of Mn(II) may be involved in the delignification of SWKP.

To clarify the role of MnP in the delignification of SWKP during treatment with *P. chrysosporium* under Mn(II)-sufficient conditions, cumulative MnP activity again was plotted against the kappa number decrease during treatment of SWKP-N with the addition of Mn(II) (Fig. 4). In the biobleaching of SWKP-N with a Mn(II) addition and HWKP, the kappa number decreased with increasing cumulative MnP activity. This suggested that MnP may be involved mostly in the delignification of SWKP under Mn(II)-sufficient conditions, although other enzymes, except MnP, may contribute to the delignification of SWKP under Mn(II)-insufficient conditions.

3.4 Delignification rate of SWKP and HWKP by MnP treatment under Mn(II)-sufficient conditions

Figures 3 and 4 show that MnP may be involved in the delignification of SWKP under Mn(II)-sufficient conditions. However, the difference in the structure of residual lignin in SWKP and HWKP should reflect the delignification rate by MnP, because Faix *et al.* reported that synthetic syringyl-guaiacyl lignins are depolymerized more rapidly than guaiacyl lignins by *P. chrysosporium*[19] and Wariishi *et al.*[20] reported that synthetic syringyl lignin is more susceptible to depolymerization by MnP than is guaiacyl lignin. Thus, MnP treatments of HWKP, SWKP-N, and SWKP-L which has almost the same kappa number as HWKP with partially purified MnP ranging from 80 to 300 U per gram of pulp under Mn(II)-added conditions were made to study whether or not there is a difference in the delignification rates of SWKPs and HWKP by *in vitro* MnP treatments. Furthermore, alkaline nitrobenzene oxidation analysis were made with SWKPs and HWKP to clarify the differences in structures of residual lignin in pulps. MnP treatment

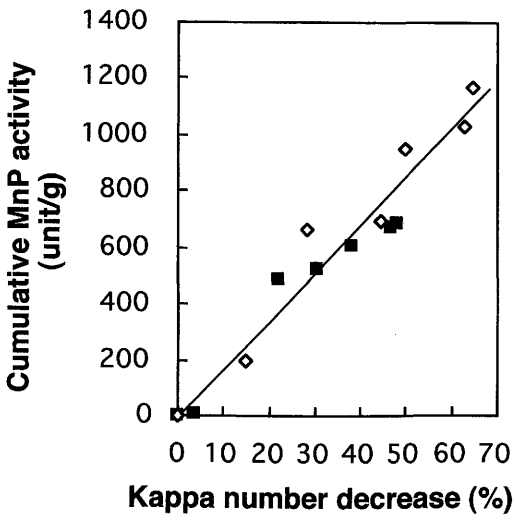

Fig. 4. Relationship between the cumulative MnP activity and kappa number decrease of UKP during treatment with *P. chrysosporium*.
Legend: ■: SWKP-N with Mn(II) addition; ◇: HWKP without Mn(II) addition.

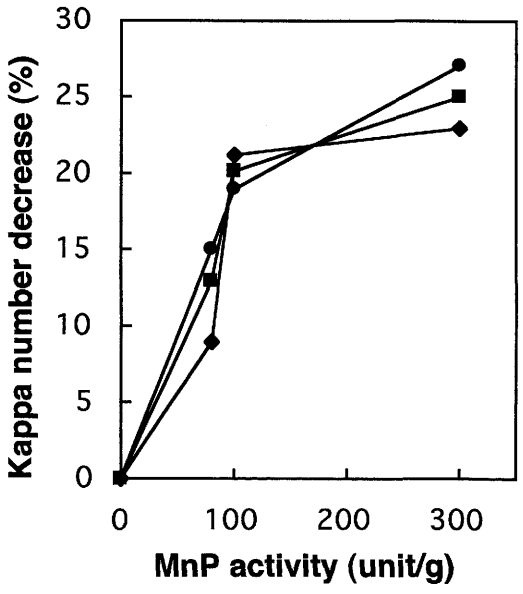

Fig. 5. Delignifications of SWKPs and HWKP by *in vitro* MnP treatments.
Legend: ◆: SWKP-N; ■: SWKP-L; ●: HWKP.

Table 4. Yields of nitrobenzene oxidation products from UKP.

Pulp	Yield (% of lignin[a])				
	Vanillin	Vanillic acid	Syringaldehyde	Syringic acid	Total
HWKP	1.65	0.50	2.52	0.60	5.27
SWKP-N	8.38	1.95	—[b]	—[b]	10.33
SWKP-L	7.29	1.87	—[b]	—[b]	9.16

[a] Lignin content was obtained by the following equation: lignin content=0.15×kappa number.
[b] Not detected.

showed no significant differences in the delignification rates between SWKPs and HWKP (Fig. 5). This result suggests that the delignification of UKPs by *in vitro* MnP treatments, in which the applied MnP activity was fixed, may be approximated as a first-order process in the amount of residual lignin. The nitrobenzene oxidation analysis revealed low yields of syringaldehyde and syringic acid from HWKP (3.12% of lignin in Table 4), indicating that the residual lignin in HWKP contains little syringyl unit and much guaiacyl unit. This may be responsible for no significant difference in the delignification rates by isolated MnP between HWKP and SWKPs.

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