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Yuka Iiyoshi · Yuji Tsutsumi · Tomoaki Nishida

Polyethylene degradation by lignin-degrading fungi and manganese peroxidase*

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Abstract Degradation of high-molecular-weight polyethylene membrane by lignin-degrading fungi, IZU-154, *Phanerochaete chrysosporium*, and *Trametes versicolor*, was investigated under various nutritional conditions. IZU-154 showed the most significant polyethylene degradation among the three lignin-degrading fungi under nitrogen- or carbon-limited culture conditions. Furthermore, for *T. versicolor* and *P. chrysosporium*, the addition of Mn(II) into nitrogen- or carbon-limited culture medium enhanced polyethylene degradation. These results suggest that polyethylene degradation is related to ligninolytic activity of lignin-degrading fungi. Treatment of polyethylene membrane with partially purified manganese peroxidase (MnP) caused significant degradation in the presence of Tween 80, Mn(II), and Mn(III) chelator. This result demonstrates that MnP is the key enzyme in polyethylene degradation by lignin-degrading fungi.

Key words Polyethylene · Biodegradation · Lignin-degrading fungi · Manganese peroxidase · Ligninolytic activity

Introduction

Many natural polymers, such as cellulose and protein, are susceptible to microbial attack. In contrast, high-molecular-weight synthetic polymers are generally difficult to degrade by microorganisms. Environmental concerns have led us to seek ways to resolve the problem that recalcitrant plastics are accumulating on earth.

It was reported that many microorganisms can utilize paraffins as a carbon source[1]; therefore, the idea that polyethylenes could also be catabolized by such microbes has been of interest. Polyethylene is one of the most abundant commercially produced synthetic polymers. Polyethylene and paraffins were first compared in degradation experiments by Jen-hou and Schwartz, who counted the number of bacteria grown on these alkanes as a measure of polyethylene utilization.[2] They showed that such microbes can grow on a low-molecular-weight polyethylene [weight-average molecular weight (*M*w) 4800], but not the higher-molecular-weight polyethylenes. Albertsson and Banhidi[3] examined the biodegradation of high-density (linear) polyethylene (HDPE) film (*M*w 93 000) for 2 years and found that the short-chain oligomeric fraction contained in HDPE film is the main degraded component.

There is currently a great interest in the lignin-degrading fungi because their industrial potentials are recognized in biomechanical pulping,[4] biobleaching,[5–7] dye decolorization,[8] and detoxification of recalcitrant environmental pollutants such as dioxins and chlorophenols.[9,10] The biodegradation of degradable plastic film made with linear low-density polyethylene-containing pro-oxidants and 6% starch by the lignin-degrading fungus *Phanerochaete chrysosporium* has been attempted, but virtually no degradation of polyethylene pretreated with 70°C heating or 365-nm ultraviolet (UV) irradiation by the fungal treatment was observed.[11] In a previous paper, one of the authors demonstrated that the lignin-degrading fungus IZU-154 significantly degrades nylon-66 and nylon-6 membranes under the ligninolytic condition,[12] suggesting that degradation of polyethylene was plausible. In this study, therefore, we examined the degradation of polyethylene membrane by lignin-degrading fungi under various nutritional conditions and investigated the enzymes related to its degradation.

Y. Iiyoshi · Y. Tsutsumi · T. Nishida (✉)
Department of Forest Resources Science, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan
Tel. & Fax: +81-54-238-4852
e-mail: aftnisi@agr.shizuoka.ac.jp

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Materials and methods

Microorganisms

Phanerochaete chrysosporium ME-446, *Trametes versicolor* IFO 7043, and IZU-154[13–16] were used in this study. Identification of IZU-154 was not completed, although this fungus may belong to the family Deuteromycotina because secondary mycelia were observed and the sexual cycle was not. IZU-154 was deposited as the strain name of NK-1148 under the accession number FERM BP-1859 with the National Institute of Bioscience and Human Technology of the Ministry of Industry and Technology, Ibaraki, Japan.

These fungi were maintained in potato dextrose agar (PDA; Difco Laboratories) slants. A new PDA plate was inoculated with each lignin-degrading fungus and precultured for 5–7 days at 30°C.

Treatment of polyethylene with lignin-degrading fungi

Each of two disks punched from the grown edge of the precultured mycelium on the PDA plate were placed on opposite sides of the agar medium containing various nutritional nitrogen and carbon concentrations. After the medium was incubated for 7 days at 30°C, two strips (1 × 6 cm, 100 μm thick) of polyethylene membrane (HIPORE-1100, Asahi Kasei) were placed on the grown mycelium and incubated statically at 30°C with lignin-degrading fungus.

Basal agar medium was prepared by dissolving 0.75 g $(NH_4)_2SO_4$, 20 g glucose, 1.0 g KH_2PO_4, 0.2 g NaH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 μg $ZnSO_4 \cdot 7H_2O$, 20 μg $CuSO_4 \cdot 5H_2O$, 100 μg $CaCl_2$, 100 μg $FeSO_4 \cdot 7H_2O$, 10 μg $MnSO_4 \cdot 4H_2O$, 30 g agar in 1 l distilled water; the pH of the medium was adjusted to 4.5. For some experiments different concentrations of $(NH_4)_2SO_4$ and glucose were used to study the effect of nitrogen and carbon concentration on polyethylene degradation. The effect of manganese sulfate on polyethylene degradation was examined using ammonium sulfate- or glucose-free medium containing 0.24 mM $MnSO_4 \cdot 4H_2O$.

Enzyme assays

To assay enzyme activity, the agar media with mycelia were homogenized using a mortar and pestle at 4°C. An aliquot of the homogenate was added to the reaction mixtures containing substrates. The mixtures were homogenized by a high-speed mixer for 30 s at 10000 rpm, and the reaction was carried out at 37°C. After the reaction the agar was immediately filtered off, and absorbance of filtrate was monitored as described below. Laccase activity was measured by monitoring the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 415 nm.[17] The reaction mixture contained 7.8 mM ABTS and 20 mM sodium succinate buffer (pH 4.5). Manganese peroxidase (MnP) activity was determined by subtracting laccase activity from the oxidation of ABTS at 415 nm[18] in the presence of 0.1 mM $MnSO_4$, 50 mM sodium lactate, and 0.1 mM H_2O_2 in the above reaction mixture. Lignin peroxidase (LiP) activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm.[19] The reaction mixture contained 10 mM veratryl alcohol, 0.1 mM H_2O_2, and 20 mM sodium succinate buffer (pH 3.0). One unit of enzyme activity is defined as the amount of enzyme that increases the absorbance by 0.1/min under the above conditions, and enzyme activity was expressed in units per gram of the agar medium.

Preparation and purification of MnP

Manganese peroxidase was prepared from the culture of *P. chrysosporium* and partially purified according to our previous report.[20] Laccase and LiP activities were not detected in the partially purified MnP.

Treatment of polyethylene with MnP

Ten strips of polyethylene membrane were added to 50 ml 50 mM malonate buffer (pH 4.5) containing 0.2 mM $MnSO_4$, 1500 units of partially purified MnP, 2 units of glucose oxidase (GOD) (from *Aspergillus niger*; Wako Pure Chemicals Industries), and 25 mM glucose. Experiments to evaluate the effect of adding 0.1% Tween 80 (Pierce Chemical Co.) on polyethylene degradation were carried out with and without GOD and glucose for supplying H_2O_2 to the MnP reaction mixture. The effect of adding manganese sulfate was examined with 0.1% Tween 80 but without GOD and glucose. Furthermore, 50 mM acetate buffer (pH 4.5) was used instead of 50 mM malonate buffer to study the effect of chelator for Mn(III) with 0.1% Tween 80 but without GOD and glucose. The reaction was conducted with shaking at 130 rpm at 37°C for 8 days in a 100-ml Erlenmeyer flask.

Evaluation of polyethylene degradation

Polyethylene degradation was followed by changes in relative elongation (the extension of the material under load), relative tensile strength (the stress measured at the fracture of the specimen), and polyethylene molecular weight distribution. Elongation and tensile strength were determined on a Strograph-R3 (Toyo Seiki) at 20°C, 60% relative humidity (RH), and 100 mm/min with a 3-cm gap. Relative elongation (%) and relative tensile strength (%) are defined as the percentage of elongation and tensile strength of fungus- or enzyme-treated polyethylene membrane compared to those of untreated membranes, respectively.

A Waters model 150-C (Waters Co.) high-temperature gel permeation chromatograph (HT-GPC) was used to determine the polyethylene molecular weight distribution. Two identical SHODEX UT-806L-linear columns (Showa Denko) were used in series. A mobile phase of *o*-dichlorobenzene (ODCB) containing 0.1% 2,6-*di-tert*-butyl-*p*-cresol (BHT) as antioxidant, a flow rate of 1 ml/min, and an injection volume of 400 μl were used. The total run time was 75 min per injection, followed by a 3-min equilibration delay. A refractive index detector was used.

Injector, columns, and detector were all held at 145°C, and the solvent pump was held at 50°C. A molecular weight calibration curve was constructed based on 12 narrow-molecular-weight distribution polystyrene standards, with peak molecular weights ranging from 794 to 20000000. Samples were prepared in ODCB containing 0.1% BHT, and they contained 0.2% (w/v) polyethylene. Initially, 16-mg polyethylene samples were added to amber jars along with 8 ml of ODCB with BHT. The jars were capped and placed in a 150°–155°C convection oven for 3 h with occasional swirling. The dissolved samples were transferred to Waters filter vials, manually filtered through the integral, Teflon-housed, sintered stainless-steel filter (0.5 μm), and immediately placed in the HT-GPC autosampler at 145°C. Duplicate injections were run from each sample. Labcharts 180 computer software (System Instruments) was used to determine the weight-average molecular weight (*M*w), number-average molecular weight (*M*n), and polydispersity (*M*w/*M*n) of the polyethylene samples.

Results and discussion

Polyethylene degradation by lignin-degrading fungi

Polyethylene degradation by lignin-degrading fungi under various nutritional conditions was investigated. Figure 1 shows the effect of nitrogen concentration on polyethylene degradation by IZU-154. The elongation and tensile strength of the polyethylene membrane were reduced drastically with the ammonium sulfate (AS)-free basal medium (N-0 medium), whereas they hardly changed with the media containing AS at 0.75, 0.50, or 0.25 g/l concentration. With an N-0 medium, relative elongation decreased by 88% after 2 days of IZU-154 treatment, with no elongation observed after 4 days. Relative tensile strength decreased by about 50% and 73% after 6 and 12 days, respectively. The elongation and tensile strength of an uninoculated control after 12 days were the same as those of the zero-time control, which was not inoculated and not incubated.

Polyethylene degradation by IZU-154 with an N-0 medium was also confirmed by the HT-GPC profiles (Fig. 2). The *M*w, *M*n, and *M*w/*M*n are shown in Table 1. The polyethylene *M*w decreased drastically from 716000 to 296000 after 2 days of treatment and reached 118000 after 12 days of treatment with IZU-154. As shown in Fig. 1, a rapid reduction in relative elongation was also observed after a 2-day treatment and the relative tensile strength decreased steadily with increasing duration of fungal treatment. These results indicate that the change in *M*w during treatment with IZU-154 is similar to that for elongation.

Polyethylene degradation by two well-known lignin-degrading fungi, *P. chrysosporium* and *T. versicolor*, was compared with that by IZU-154 in AS-free (N-0) culture (Fig. 3). *P. chrysosporium* produced the same level of degradation as IZU-154, but *T. versicolor* degraded polyethylene less than the other two fungi.

Figure 4 shows the effect of carbon concentration on polyethylene degradation by IZU-154. Reductions in elongation and tensile strength were observed in the medium containing glucose at less than 0.5 g/l. The most significant reduction was obtained with glucose-free basal (C-0) medium. With this medium, IZU-154 produced the most

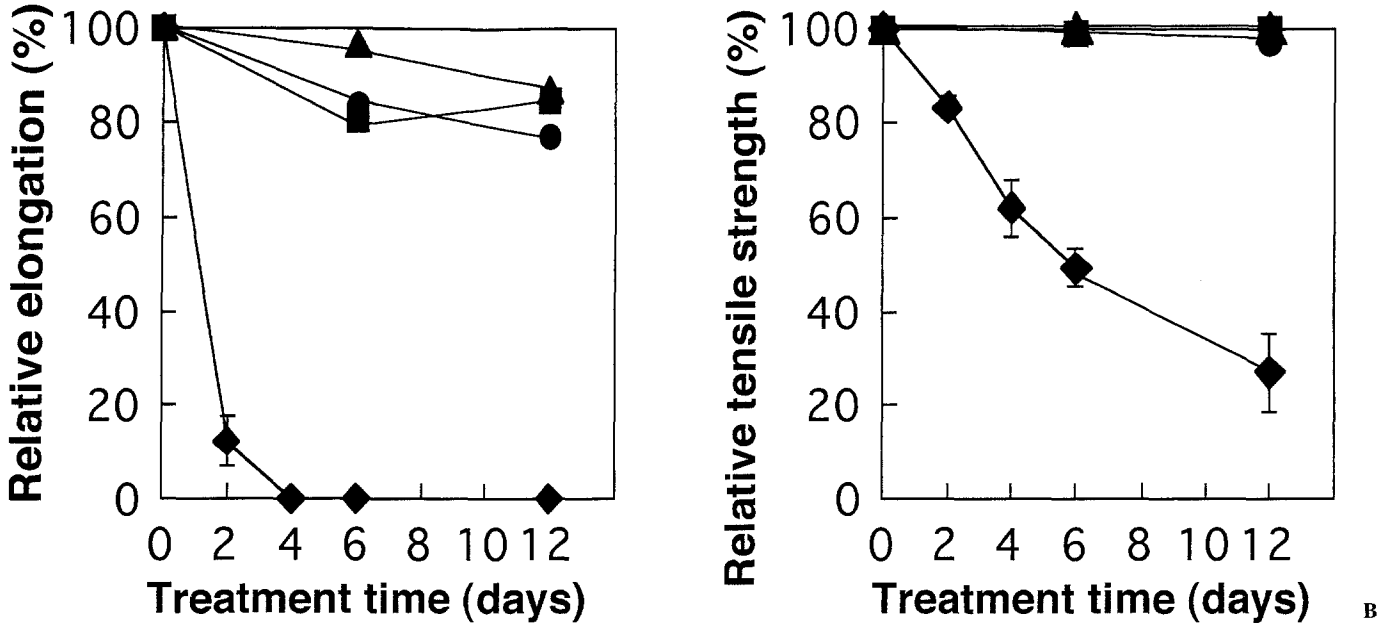

Fig. 1. Effect of ammonium sulfate (AS) concentration in the medium on reductions in relative elongation (**A**) and relative tensile strength (**B**) of polyethylene membrane by IZU-154. *Squares*, 0.75 g/l; *circles*, 0.5 g/l; *triangles*, 0.25 g/l; *diamonds*, 0 g/l (N-0 medium). The datum points are the averages of 10 replicate polyethylene strips. The deviations of the actual values from the averages are shown as bars

marked reduction in elongation and tensile strength among the three lignin-degrading fungi, followed by *P. chrysosporium* and *T. versicolor* (data not shown). However, these reductions in elongation and tensile strength with a C-0 medium were lower than those with an N-0 medium. This result indicates that nutritional nitrogen limitation affords more effective polyethylene degradation by lignin-degrading fungi. Ligninolytic activity of lignin-degrading fungi appears as a secondary metabolic event, and nutritional nitrogen or carbon limitation (or both) allows extensive degradation of lignin.[21–23] Therefore, it is likely that ligninolytic activity of lignin-degrading fungi is related to polyethylene degradation.

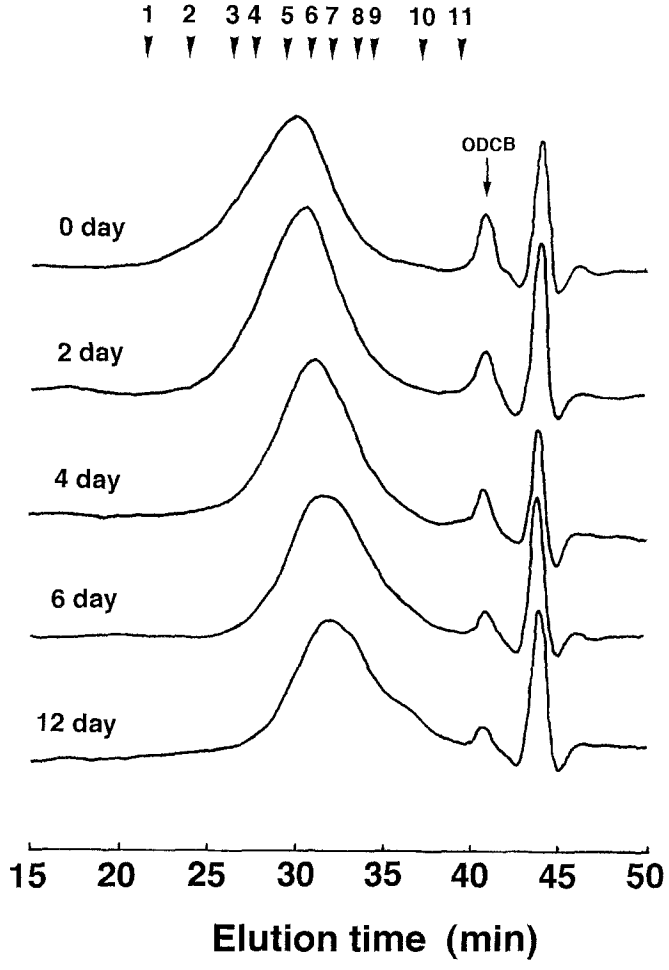

Fig. 2. High-temperature gel permeation chromatography (HT-GPC) analyses of polyethylene membrane treated with IZU-154 under AS-free culture condition. *ODCB*, *o*-dichlorobenzene. *Arrows* indicate the elution times of polystyrene standards: *1* (*M*w 200 × 10^5); *2* (*M*w 258 × 10^4); *3* (*M*w 103 × 10^4); *4* (*M*w 676 × 10^3); *5* (*M*w 344 × 10^3); *6* (*M*w 181 × 10^3); *7* (*M*w 984 × 10^2); *8* (*M*w 439 × 10^2); *9* (*M*w 189 × 10^2); *10* (*M*w 510 × 10^1); *11* (*M*w 794)

It has been suggested that laccase, MnP, and LiP produced extracellularly by lignin-degrading fungi are involved in the oxidative breakdown of lignin.[24–30] Therefore, these enzyme activities were determined during treatment of polyethylene membrane with IZU-154, *P. chrysosporium*, and *T. versicolor* with an N-0 medium (Table 2). All fungi produced laccase and MnP. In contrast, LiP activity was not detected during treatment of polyethylene membrane, which was confirmed by five experiments, though it has been widely reported that *P. chrysosporium* produces LiP on low nitrogen medium. With an N-0 medium, the laccase activity produced by *T. versicolor* was much higher than that by IZU-154 or *P. chrysosporium*; no significant polyethylene degradation was obtained with *T. versicolor* (Fig. 3). This result suggests that laccase is not involved in polyethylene degradation. On the other hand, IZU-154 and *P. chrysosporium*, which showed a higher potential for polyethylene degradation than *T. versicolor* in N-0 medium, produced much more MnP than *T. versicolor*. As shown in Fig. 5, polyethylene degradation by *T. versicolor* was accelerated by the addition of 0.24 mM manganese sulfate to an N-0 medium, and this fungus showed almost the same degradation ability as IZU-154 and *P. chrysosporium* with an N-0 medium in which Mn(II) was not added. Simultaneously, MnP production was accelerated by the addition of Mn(II) to an N-0 medium (Table 2). It was further confirmed that the addition of Mn(II) to a C-0 medium also enhanced polyethylene degradation by *T. versicolor* and *P. chrysosporium* (data not shown). For lignin degradation by lignin-degrading fungi, manganese is an important cofactor, especially as a regulator of MnP production and an active mediator in the MnP catalytic cycle.[21,31–35] It is obvious that manganese plays an important role in the polyethylene degradation system, and MnP may be involved in the degradation by lignin-degrading fungi in a culture containing sufficient manganese.

Table 1. Changes in molecular weight (weight-average and number-average) of polyethylene membrane during treatment with IZU-154 on N-0 medium

Parameter	Changes, according to incubation time (0–12 days)				
	0	2	4	6	12
*M*w	716000	296000	194000	150000	118000
*M*n	108000	87300	63200	37000	23600
*M*w/*M*n	6.63	3.39	3.07	4.05	5.00

Values are averages of 10 replicate polyethylene strips. Duplicate high-temperature gel permeation chromatography (HT-GPC) runs were performed for each strip.
*M*w, weight-average molecular weight; *M*n, number-average molecular weight.

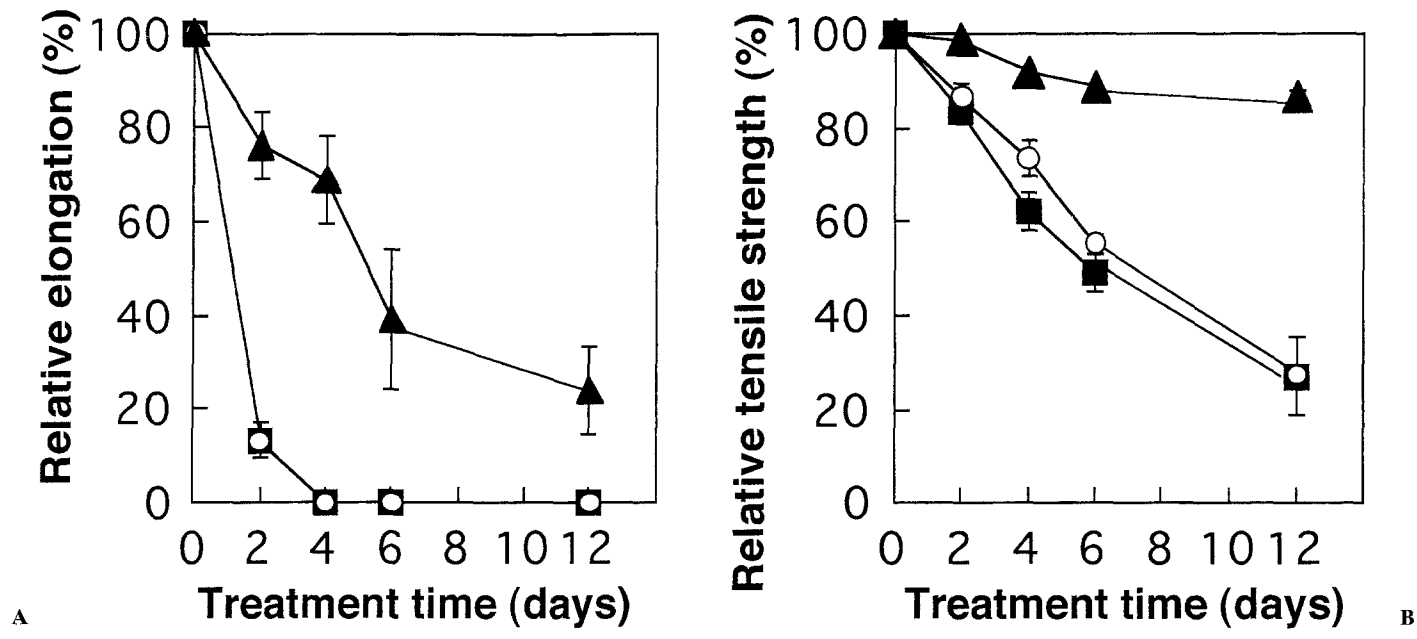

Fig. 3. Reductions in relative elongation (**A**) and relative tensile strength (**B**) of polyethylene membrane by lignin-degrading fungi with N-0 medium. *Squares*, IZU-154; *circles*, *P. chrysosporium*; *triangles*, *T. versicolor*. Refer to Fig. 1

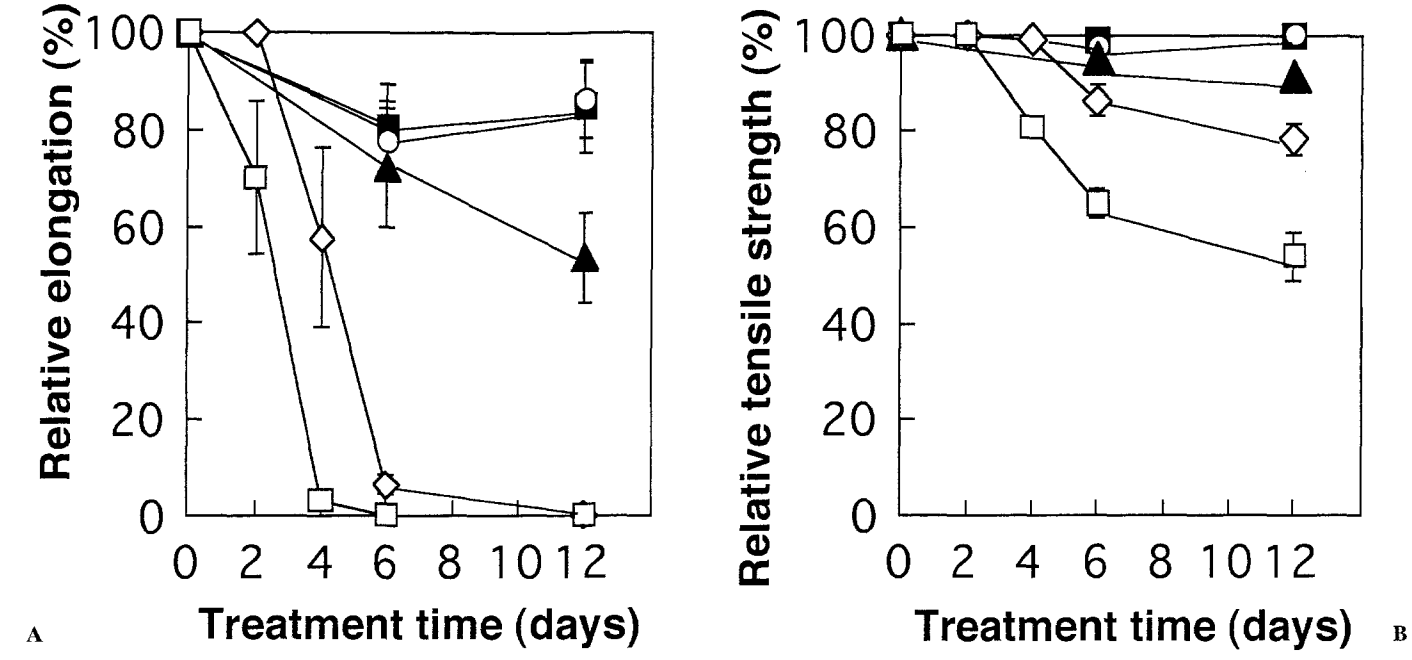

Fig. 4. Effect of glucose concentration in the medium on reductions in relative elongation (**A**) and relative tensile strength (**B**) of polyethylene membrane by IZU-154. *Filled squares*, 20 g/l; *circles*, 5 g/l; *triangles*, 1 g/l; *diamonds*, 0.5 g/l; *open squares*, 0 g/l (C-0 medium). Refer to Fig. 1

Polyethylene degradation by MnP

Studies on Mn(II) addition during fungal treatment encouraged us to expect polyethylene degradation by MnP, so treatment of the polyethylene membrane with MnP was examined. In this experiment, the effect on polyethylene degradation of adding 0.1% Tween 80 was examined with or without GOD and glucose for supplying H_2O_2 because Kondo et al. reported that addition of a surfactant such as Tween 80 can accelerate the degradation of residual lignin in pulp.[27] As shown in Fig. 6, the addition of Tween 80 accelerated the reduction in elongation and tensile strength, and the same level of polyethylene degradation was obtained with or without GOD and glucose. These results

Table 2. MnP and laccase activities produced by IZU-154, *P. chrysosporium*, and *T. versicolor* during treatment of polyethylene membrane

Incubation time (days)	IZU-154	*P. chrysosporium*	*T. versicolor*	
	N-0 medium	N-0 medium	N-0 medium	N-0 medium + Mn(II)
MnP activity				
0	18.8 ± 3.7	16.7 ± 0.1	0	4.2 ± 1.3
2	19.8 ± 0.6	11.2 ± 0.1	0	6.6 ± 1.5
4	29.6 ± 0.9	26.3 ± 0.9	0	6.6 ± 1.4
6	17.8 ± 1.7	18.0 ± 0.5	4.9 ± 2.9	12.5 ± 0.6
Laccase activity				
0	0.8 ± 0	0.2 ± 0	6.1 ± 0.8	1.9 ± 0.8
2	0.1 ± 0	0.3 ± 0	2.4 ± 0.5	3.8 ± 0.8
4	0.1 ± 0	0.2 ± 0.1	4.7 ± 0.4	5.2 ± 0.9
6	0.7 ± 0.2	0.5 ± 0.1	1.5 ± 0	10.9 ± 3.2

Values are averages of five experiments ± deviations.

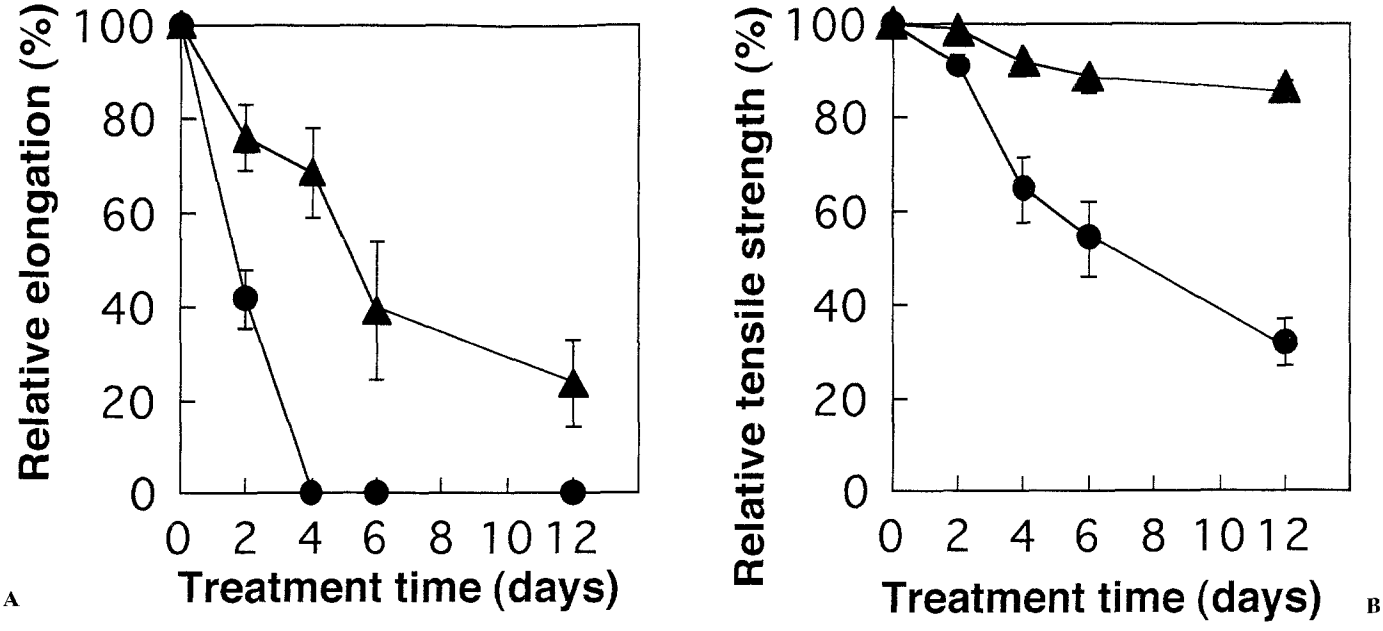

Fig. 5. Effect of manganese sulfate [Mn(II)] on reductions in relative elongation (**A**) and relative tensile strength (**B**) of polyethylene membrane by *T. versicolor*. *Triangles*, N-0 medium; *circles*, Mn(II)-supplemented N-0 medium. Refer to Fig. 1

indicate that Tween 80 is important and H_2O_2 supply is not necessary for polyethylene degradation by MnP.

For the next step, polyethylene membrane was treated with MnP under conditions that included 0.1% Tween 80 but did not supply exogenous H_2O_2; it was then analyzed by HT-GPC. As shown in Table 3, polyethylene degradation evidently accelerated after addition of manganese sulfate. The maximal effect was obtained with 0.1 mM $MnSO_4$; there was no elongation, and the *M*w was reduced from 716000 to 89500. The effects of other metals (0.1 mM $FeSO_4$, $ZnSO_4$, and $CuSO_4$) were negligible (data not shown). Furthermore, polyethylene membrane was not degraded when 50 mM acetate buffer, instead of malonate buffer, was used. It is well known that malonate can chelate with Mn(III) generated from Mn(II) in the MnP catalytic cycle. This result supports the idea that Mn(III) is closely related to polyethylene degradation. From the results obtained here, it is clear that polyethylene can be degraded by MnP in the presence of Tween 80, $MnSO_4$, and a buffer having a potential for the formation of Mn(III) chelate. However, the addition of exogenous H_2O_2 was not necessary for the degradation, although it is recognized that H_2O_2 is essential for the MnP reaction system. Moen et al. have reported that MnP promotes the peroxidation of unsaturated fatty acid (lipid), such as Tween 80, in the presence of Mn(II) and oxygen but without exogenous H_2O_2, though it is not clear what role Mn(III) plays in this system. Moen et al. also proposed that the radicals generated from the lipid peroxidation can react to lignin and polycyclic aromatic hydrocarbon as active oxidants.[36,37] At least part of the

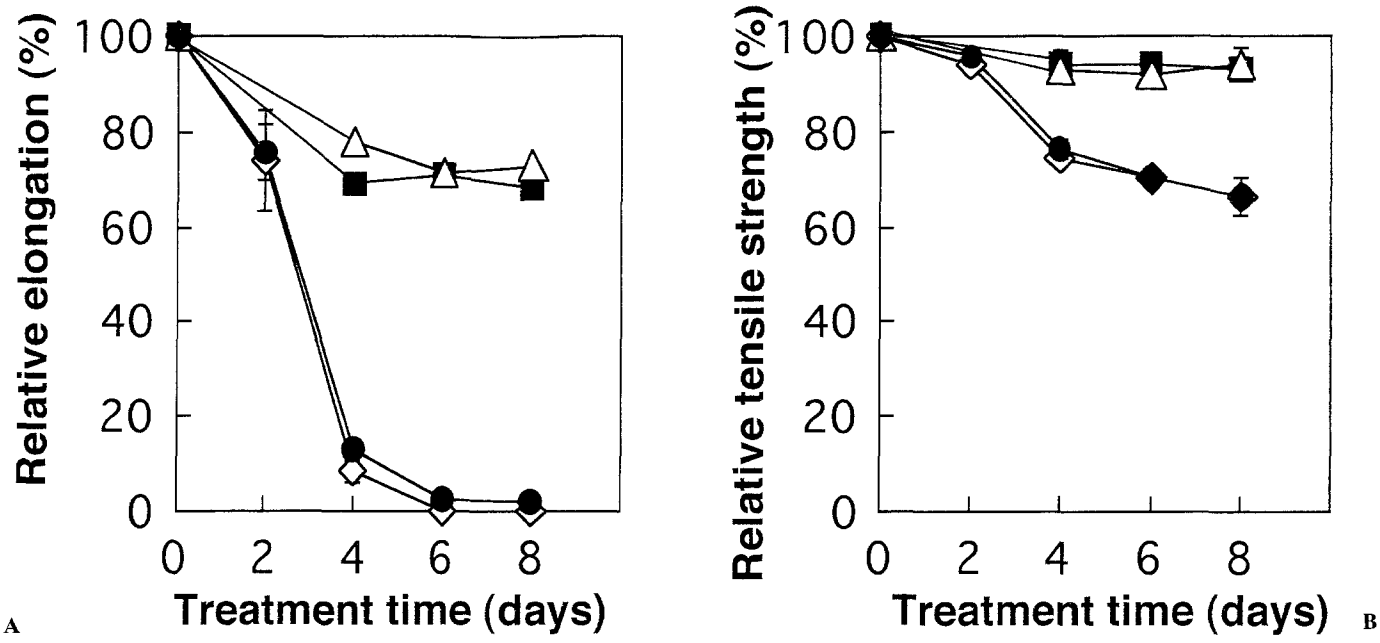

Fig. 6. Effect of Tween 80 addition to the reaction mixture on reductions in relative elongation (**A**) and relative tensile strength (**B**) of polyethylene membrane during treatment with manganese peroxidase (MnP). *Circles*, with Tween 80, glucose oxidase (GOD), and glucose; *diamonds*, with Tween 80, without GOD and glucose; *triangles*, without Tween 80, GOD, and glucose; *squares*, without Tween 80, with GOD and glucose. Refer to Fig. 1. Control experiment was performed without adding MnP

Table 3. Effect of Mn(II) concentration in the reaction mixture on degradation of polyethylene membrane by MnP treatment for 8 days

Polyethylene membrane	Relative elongation (%)	Relative tensile strength (%)	*Mw*[a]	*Mn*[a]	*Mw*/*Mn*[a]
Untreated control	100.0 ± 6.9	100.0 ± 3.4	716000	108000	6.63
MnP treatment					
0.2 mM $MnSO_4$ and 50 mM malonate	0	75.6 ± 3.9	113000	56000	2.02
0.1 mM $MnSO_4$ and 50 mM malonate	0	52.5 ± 9.4	89500	38800	2.31
0.01 mM $MnSO_4$ and 50 mM malonate	84.1 ± 6.0	97.7 ± 3.7	489000	112000	4.01
0.2 mM $MnSO_4$ and 50 mM acetate	91.2 ± 9.0	100.0 ± 1.3	ND	ND	ND

Values are averages of 10 replicate polyethylene strips ± deviations.
ND, not determined.
[a]Duplicate HT-GPC runs were performed for each strip.

result, in which the addition of Tween 80-containing unsaturated fatty acid esters brought about marked polyethylene degradation without exogenous H_2O_2, probably is explained by the MnP–lipid peroxidation system proposed by Moen et al. There is another possible mechanism: H_2O_2, which is essential for the MnP reaction system, is supplied by lipid autoxidation and following reactions, including a superoxide dismutation. The roles of Tween 80 and oxygen relevant to the MnP catalytic cycle and polyethylene degradation comprise the subject of a forthcoming study.

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