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Degradation of polyethylene and nylon 66 by the laccase-mediator system

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ABSTRACT

We investigated whether the laccase-mediator system (LMS) with 1-hydroxybenzotriazol (HBT) as a mediator could degrade high-molecular-weight polyethylene and nylon-66 membranes. The LMS markedly reduced the elongation and tensile strength of these membranes. After 3 days of treatment with the LMS, the *M*w of polyethylene decreased from 242,000 to 28,300, and that of nylon-66 *M*w from 79,300 to 14,700. The LMS also decreased the polydispersity (*M*w/*M*n) of polyethylene and nylon 66. Furthermore, these reductions in elongation, tensile strength, and molecular weight were accompanied by morphological disintegration of the polyethylene and nylon-66 membranes. These results strongly suggest that the LMS with HBT can effectively degrade polyethylene and nylon 66.

KEY WORDS: Polyethylene; nylon 66; biodegradation; laccase; mediator.

INTRODUCTION

High-molecular-weight synthetic polymers, such as polyethylene and nylon, are generally difficult to degrade using microorganisms. Environmental concerns about the accumulation of such plastics have led researchers to seek ways to resolve the problem.

Previously, we demonstrated that the hyperligninolytic fungus IZU-154 [1-4] considerably degrades polyethylene and nylon-66 membranes in nitrogen-free or carbon-free cultures. We also showed that these membranes and nylon-6 fibers are degraded by manganese peroxidase (MnP), a heme peroxidase produced by ligninolytic fungi that oxidize phenolic compounds in the presence of Mn(II) [5-8]. On the other hand, the well-known ligninolytic fungus *Trametes versicolor* degrades polyethylene and nylon, although this fungus is less effective than IZU-154 [5, 7]. After 4 days of treatment with *T. versicolor* in nitrogen-free medium, the relative elongation and tensile strength of polyethylene membrane decreased by about 30 and 10%, respectively. In addition, over 4 days of treatment, this fungus produced laccase, but no other ligninolytic enzymes (MnP and lignin peroxidase [LiP]) [5], which suggests that laccase is also involved in polyethylene degradation.

Fungal laccase is a blue, multicopper-containing enzyme that catalyzes one-electron oxidation of phenolic compounds by reducing molecular oxygen to water [9]. In the presence of a mediator such as 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) or 1-hydroxybenzotriazol (HBT), laccase can oxidize nonphenolic compounds that it

cannot oxidize alone [10, 11]. Recently, this laccase-mediator system (LMS) has been used to oxidatively break down lignin [12] and some environmental pollutants (e.g., phosphorothiolates [13], polycyclic aromatic hydrocarbons [14, 15], and estrogenic chemicals [16]), although the underlying mechanisms of these reactions remain unclear. The results of these studies have led us to predict that the LMS can degrade polyethylene and nylon, because laccase can degrade a wider range of substrates in the presence of a mediator.

In this study, we treated polyethylene and nylon 66 with the LMS using HBT as a mediator, and described the degradation of their synthetic polymers.

EXPERIMENTAL DESIGN

Materials

We used polyethylene membrane (HIPORE-1100; Asahi Kasei) and nylon-66 membrane (Sartolon Polyamid; Sartorius) in this study. All other chemicals were extra pure grade, obtained from commercial sources.

Laccase assay

Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 470 nm. The reaction mixture contained 1 mM DMP and 50 mM malonate buffer (pH 4.5). One kat of laccase activity was defined as the amount of

enzyme that produces 1 mol quinone dimer from DMP per second [17].

Preparation and purification of laccase

We inoculated *T. versicolor* IFO 6482 onto potato dextrose agar plates and incubated them for 7 days at 30°C. Four disks punched from the growing edge of the mycelium were homogenized for 15 s with 50 ml GP medium (per liter distilled water: 30 g glucose, 10 g peptone, 1.5 g KH2PO4, 0.5 g MgSO4·7H2O, 16 mg CuSO4·5H2O, and 2 mg thiamine hydrochloride; pH 4.5). The homogenate was placed in a 500-ml Erlenmeyer flask with 150 ml GP medium and shaken at 150 rpm at 30°C. After 13 days, 50 ml of the culture was homogenized again and cultured in a 500-ml Erlenmeyer flask with 150 ml of GP medium for 8 days on a rotary shaker (150 rpm; 30°C). The mycelium was removed by centrifugation; ammonium sulfate (AS) was then added to the supernatant (about 5000 ml), and the AS was fractionated at 30 to 90% saturation. After standing overnight, the precipitate was collected by centrifugation, dissolved in a minimal volume of distilled water, and dialyzed overnight against water. The laccase solution was concentrated about 100-fold by ultrafiltration (pore size = a molecular weight of 10,000; Advantech) and the concentrate was loaded onto a Sephacryl S200 HR column (Amersham Pharmacia Biotech). The column was equilibrated and eluted with 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.0). After this dialysis, the partially purified laccase was free of other ligninolytic enzymes (such as MnP and LiP).

Treatment of polyethylene and nylon-66 membrane with laccase

Ten strips of polyethylene membrane (1 × 6 cm, 100 μm thick) were added to 50 ml of 50 mM malonate buffer (pH 4.5) containing 500 nkat partially purified laccase and 0.2 mM 1-hydroxybenzotriazol (HBT). Different concentrations of laccase and the mediator, HBT, were used for some experiments, to study the effect of concentration on polyethylene degradation. The reaction was conducted with shaking at 150 rpm at 30°C for 1 day in a 100-ml Erlenmeyer flask. Laccase and HBT were added to the flask every day to repeatedly treat the polyethylene membrane. Nylon-66 membrane (1 × 4 cm, 100 μm thick) was treated with 500 nkat laccase and 0.2 mM HBT at 30°C.

Evaluation of polyethylene and nylon-66 membrane degradation

Degradation of the polyethylene and nylon-66 membranes was followed by changes in relative elongation (extension of the material under load), relative tensile strength (stress measured at the fracture of the specimen), and distribution of molecular weight. Elongation and tensile strength were determined on a Strograph-R3 (Toyo Seiki) at 20°C at 60% relative humidity (RH). Polyethylene membrane was tested at a rate of 100 mm/min using a 3-cm gap, and nylon-66 membrane at 10 mm/min with a 2-cm gap. Relative elongation (%) and relative tensile strength (%) were defined as the elongation or tensile strength of LMS-treated membranes divided by that of untreated membranes, multiplied by 100.

A high-temperature gel permeation chromatograph (HT-GPC; model 150C, Waters

Co.) was used to determine the molecular weight distribution of polyethylene. Two identical columns (TSKgel, Tosho) and one unique column (SHODEX AT-807S, Showa Denko) were used in tandem, with a mobile phase of 1,2,4-trichlorobenzene (TCB) lacking antioxidant, a flow rate of 1 ml/min, and an injection volume of 200 µl. The total run time was 107 minutes per injection with no equilibration delay. A refractive index detector was used. The injector, columns, and detector were all maintained at 140°C, and the solvent pump at 40°C. A molecular weight calibration curve was constructed, based on 12 different polystyrene standards for molecular weight distribution, with peak molecular weights from 1,000 to 2,000,000. Samples contained 0.133% (w/v) polyethylene membrane in TCB. Initially, 20-mg polyethylene samples and 15 ml TCB were added to amber jars. The jars were capped, placed in a 140°C convection oven for 1 h, and swirled occasionally. The dissolved samples were transferred to Waters filter vials, manually filtered (through an integral, Teflon-housed, sintered 0.5-µm stainless-steel filter), and placed immediately in the HT-GPC autosampler maintained at 140°C. Duplicate injections were run per sample. Maxima 820 computer software (Waters Co.) was used to determine the weight-averaged molecular weight (*M*w), number-averaged molecular weight (*M*n), and polydispersity (*M*w/*M*n) of the polyethylene samples.

Nylon-66 samples (25 mg) were dissolved in hexafluoroisopropanol (HFIP; 10 ml) containing 10 mM trifluoroacetate and subjected to GPC using an HLC-8020 (Tosho) equipped with SC-8020 computer software (Tosho) to determine changes in molecular

weight distribution. Two identical TSKgel GMHHR-H columns (Tosho), one TSKgel G1000HHR column (Tosho), and a refractive index detector were used with HFIP that contained 10 mM trifluoroacetate as the mobile phase, at a flow rate of 0.6 ml/min, column temperature of 40°C, and injection volume of 50 µl. *M*w, *M*n, and *M*w/*M*n were calculated using results obtained from 11 polymethylmethacrylate standards for molecular weight distribution, with peak molecular weights from 18 to 1,520,000.

RESULTS AND DISCUSSION

Degradation of polyethylene membrane with the LMS

We investigated whether high-molecular-weight polyethylene membrane could be degraded by laccase with or without a mediator (HBT). Figure 1 shows the effect of HBT concentration on polyethylene degradation. The relative elongation of polyethylene membrane decreased by about 20% after 3 days of laccase treatment (500 nkat) without HBT, suggesting that degradation of polyethylene by laccase itself is possible. On the other hand, the elongation and tensile strength of the membrane were reduced drastically when HBT was added to the reaction mixture containing laccase (500 nkat). Adding 0.2 mM HBT to the laccase reaction mixture had the greatest effect; after 3 days of treatment, polyethylene membrane exhibited no elongation and its relative tensile strength had decreased by about 60% (Fig. 1). Elongation and tensile strength were reduced less by 2 mM HBT than by 0.2 mM HBT (Fig. 1). The elongation and tensile strength of the

treatment control (treated with 0.2 mM HBT without laccase) and the zero-time control (treated with neither laccase nor HBT) were the same after 3 days.

The redox potential of ligninolytic enzymes is thought to play a crucial role in lignin degradation using white-rot fungi, because nonphenolic subunits, the most predominant lignin substructures in wood, have high redox potentials. The redox potential of laccase is thought to be too low to oxidize a nonphenolic lignin substructure. However, laccase can oxidize nonphenolic lignin model compounds in the presence of a redox mediator such as HBT or ABTS [10, 11, 18]. Kaichang *et al*. proposed that when HBT is oxidized by laccase, the free radicals that are generated can actively oxidize nonphenolic lignin model compounds [18]; Bourbonnais *et al*. recently showed that the redox potential of HBT radicals and laccase is 878 and 585 mV, respectively [19]. These studies suggest that HBT radicals are responsible for polyethylene degradation, and that the difference in redox potential between HBT radicals and laccase may be why degradation is greater with the LMS than with laccase alone. As shown in Fig. 1, the degradation of polyethylene membrane using the LMS was inhibited by a high HBT concentration (2 mM). This inhibition may be a result of the overproduction of HBT radicals, which causes laccase to become inactive.

Figure 2 shows the effect of laccase concentration on polyethylene degradation by the LMS containing 0.2 mM HBT. Elongation and tensile strength were reduced by similar amounts in membranes treated with 500 and 1000 nkat laccase; thus, 500 nkat of laccase

activity is probably sufficient for degradation. Reduction of these characteristics by LMS was accompanied by morphological disintegration of polyethylene membrane after 5 days of treatment with 500 nkat laccase and 0.2 mM HBT (Fig. 3); therefore, we were able to determine the elongation and tensile strength of the membrane after 3 days of treatment. The effects of reaction temperature (30°C, 40°C, and 50°C) were negligible (data not shown).

Degradation of polyethylene by the LMS (500 nkat laccase and 0.2 mM HBT at 30°C) was also confirmed by HT-GPC analysis. Table 1 shows the *M*w, *M*n, and *M*w/*M*n data. The *M*w of polyethylene decreased from 242,000 to 44,000 after 2 days of LMS treatment, reaching 28,300 after 3 days; the *M*n decreased from 36,600 to 12,100 after 3 days of treatment. The LMS-treated membranes exhibited reduced polydispersity (*M*w/*M*n), which signifies a narrowing of the overall distribution of molecular weights (Fig. 4). As shown in Fig. 2, relative elongation decreased rapidly after 2 days of treatment, and relative tensile strength decreased steadily with increasing treatment duration. These results indicate that treatment with the LMS affects *M*w and elongation similarly.

Degradation of nylon-66 membrane with the LMS

Nylon-66 membrane was treated with the LMS under conditions of optimum polyethylene degradation (500 nkat laccase and 0.2 mM HBT at 30°C). The relative elongation and tensile strength of nylon-66 membrane decreased by 87 and 39%,

respectively, after 1 day of treatment (Table 2). Furthermore, nylon 66 began to disintegrate after 2 days of treatment (Fig. 5). Figure 6 shows the GPC profiles of nylon-66 membrane before and after treatment. High-molecular-weight fractions of nylon 66 were degraded selectively; the molecular weight of nylon 66 at the maximum GPC peak decreased with increasing treatment duration. After 3 days of treatment, nylon *M*w decreased from 79,300 to 14,700, and nylon *M*n from 17,300 to 4,900 (Table 2). These results strongly suggest that nylon 66 can also be degraded using the LMS.

CONCLUSIONS

High-molecular-weight synthetic polymers, including polyethylene and nylon, are of increasing environmental concern because, in general, they cannot be degraded by microorganisms. Here, we present data that an LMS containing laccase and HBT effectively degrades polyethylene and nylon 66. Further investigation is needed to verify the mechanism by which the LMS degrades these synthetic polymers.

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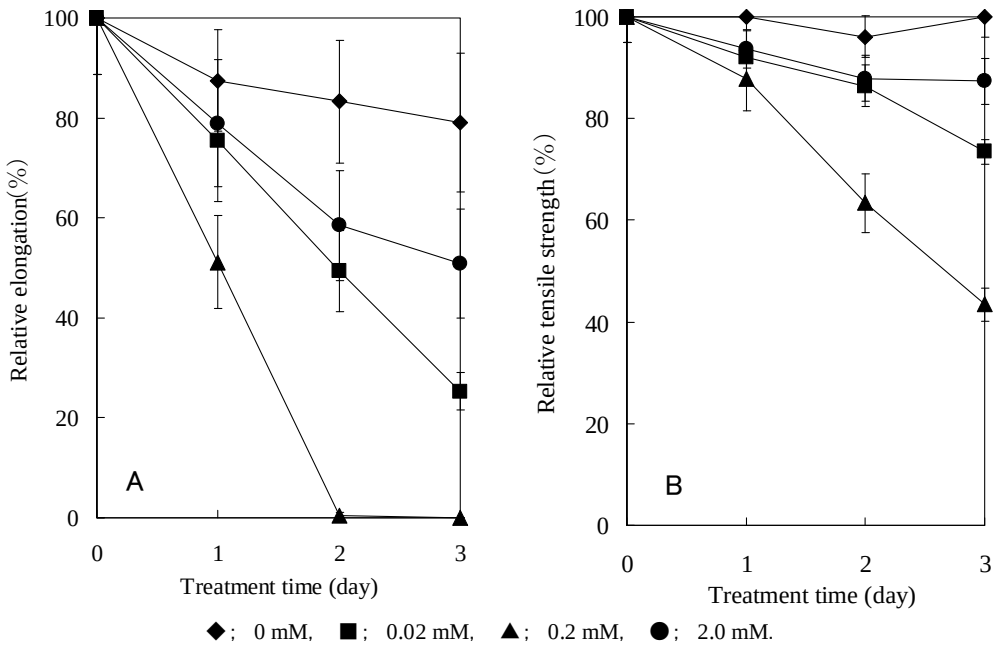

Fig. 1. Effect of HBT concentration on the reduction of relative elongation (A) and relative tensile strength (B) of polyethylene membrane. Each datum is the average of 10 replicate polyethylene strips. Deviations of the actual values from the averages are shown as bars.

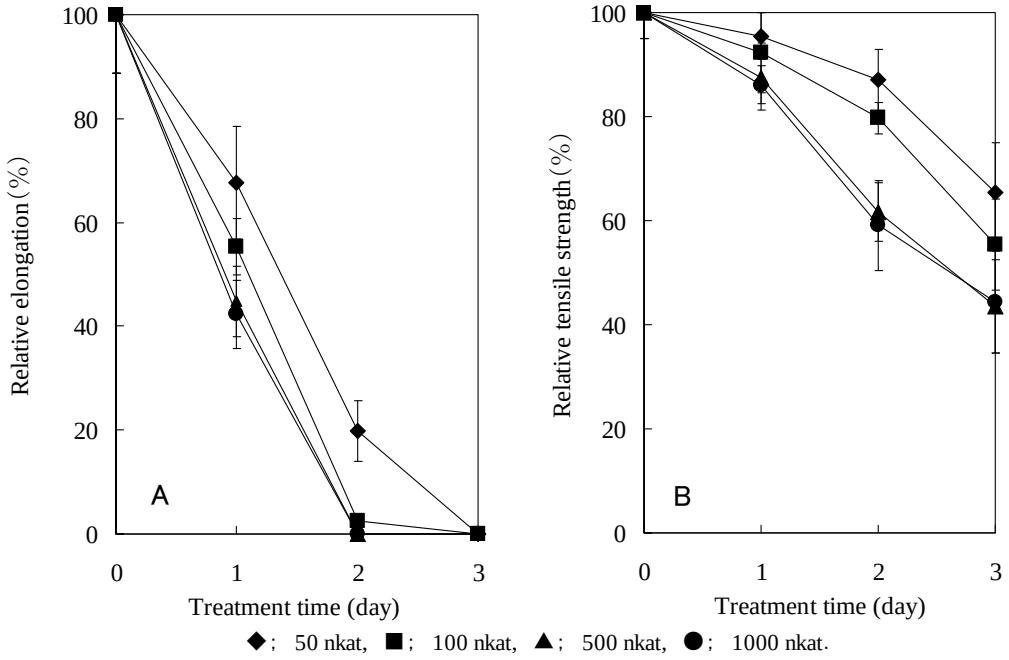

Fig. 2. Effect of laccase concentration on the reduction of relative elongation (A) and relative tensile strength (B) of polyethylene membrane. Refer to Fig. 1.

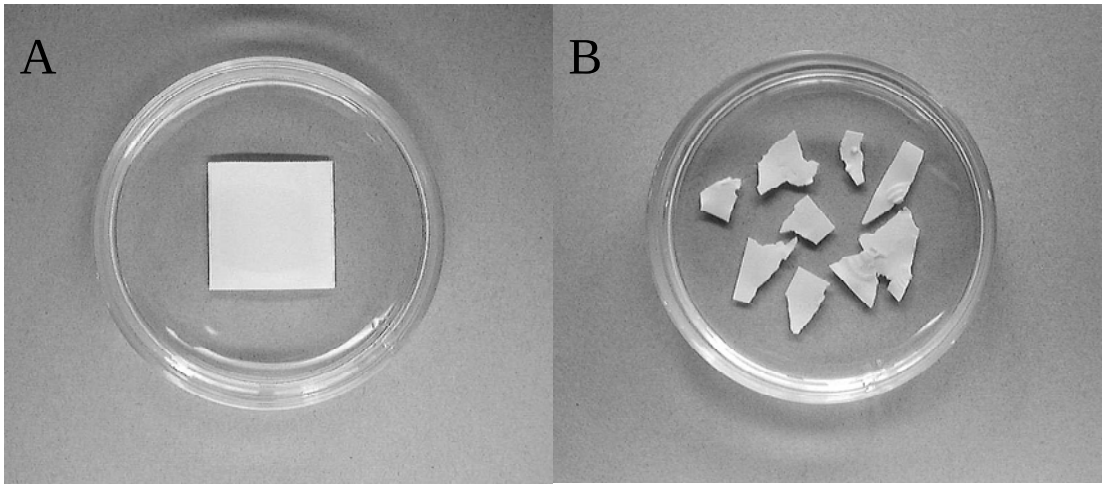

Fig. 3. Morphological disintegration of polyethylene membrane. (A) Untreated control membrane. (B) Membrane after 5 days of LMS treatment.

Table I. Changes in molecular weight (weight-average and number-average) of polyethylene membrane during LMS treatment.

Parameter	Treatment time (day)			
	0	1	2	3
*M*w	242000	135000	44000	28300
*M*n	36600	32000	17000	12100
*M*w／*M*n	6.61	4.22	2.59	2.34

Duplicate HT-GPC runs were performed for a represent strip.

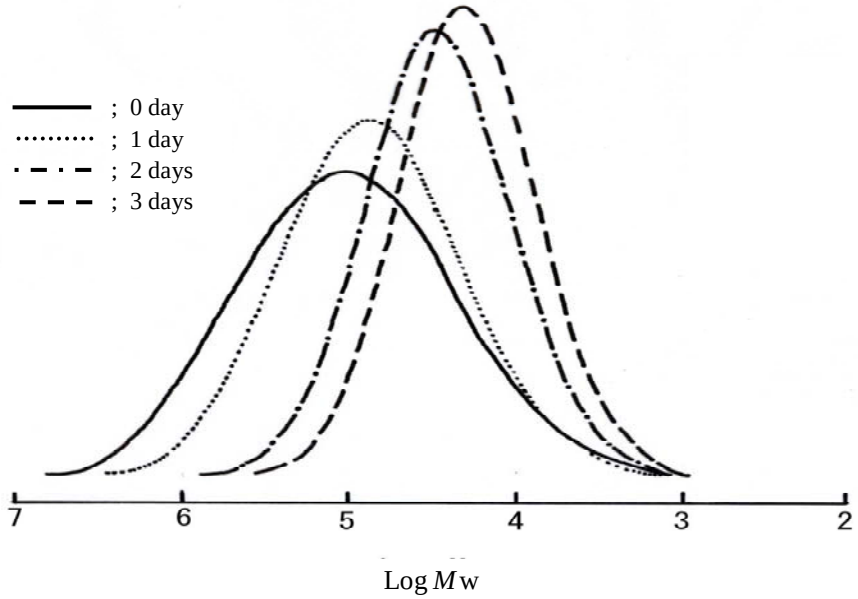

Fig. 4. HT-GPC profiles of polyethylene membrane before and after LMS treatment.

Table II. Changes in relative elongation, relative tensile strength, and molecular weight of nylon-66 membrane during LMS treatment.

Parameter	Treatment time (day)			
	0	1	2	3
Ralative elongation (%)[a]	100.0±5.0	12.9±4.2	ND	ND
Ralative tensile strength (%)[a]	100.0±4.2	61.5±3.3	ND	ND
*M*w[b]	79300	24800	17200	14700
*M*n[b]	17300	8400	6600	4900
*M*w／*M*n[b]	4.58	2.95	2.61	3.00

ND, not determined because of morphological disintegration of nylon-66 strip.

[a] Values are averages of 10 replicate nylon-66 strips ± deviations.

[b] Duplicate HT-GPC runs were performed for a represent strip.

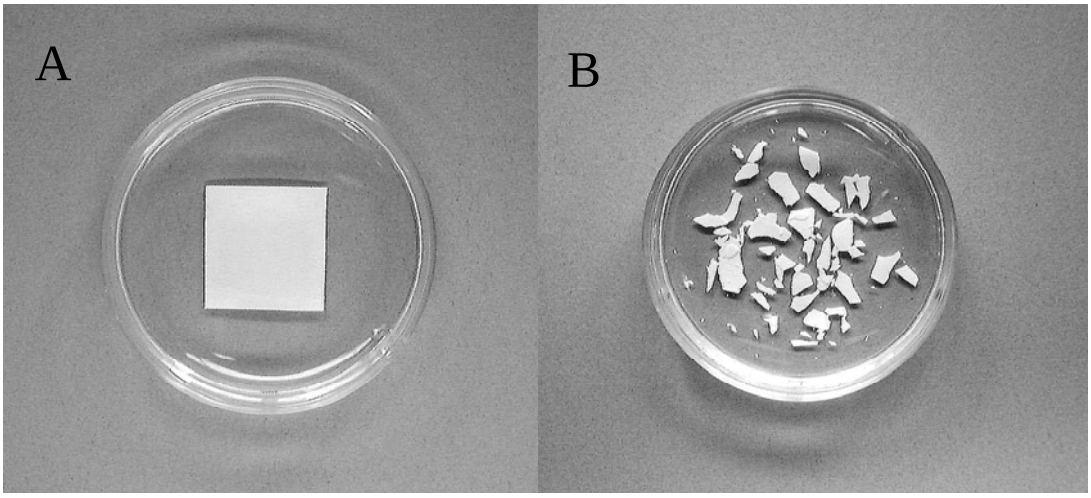

Fig. 5. Morphological disintegration of nylon-66 membrane. (A) Untreated control membrane. (B) Membrane after 2 days of LMS treatment.

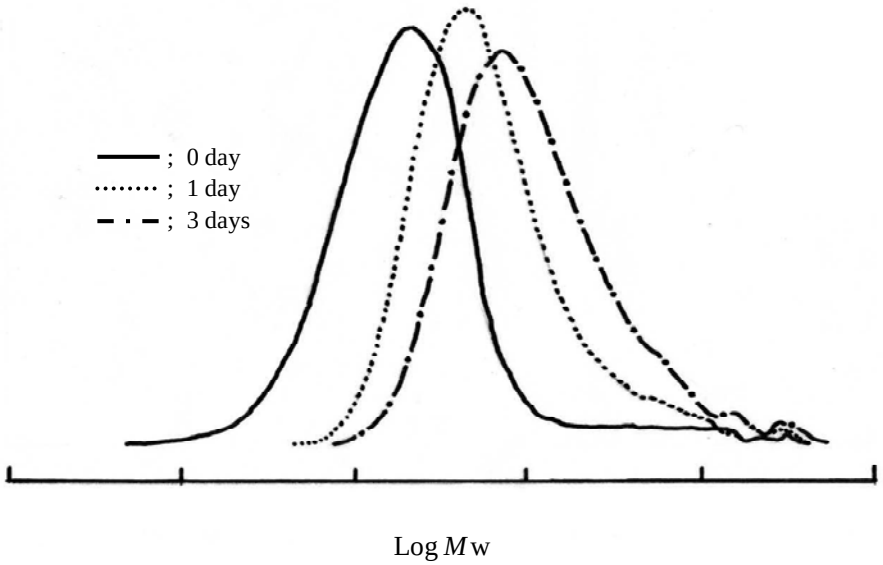

Fig. 6. HT-GPC profiles of nylon-66 membrane before and after LMS treatment.