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# Preparation of amphiphilic lignin derivative as a cellulase stabilizer

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**Abstract** A polymeric amphiphile, PE-AL, was prepared from acetic acid lignin (AL) obtained by acetic acid pulping of birch under atmospheric pressure with polyethylene glycol diglycidyl ether (PE) as the crosslinker. The behavior of PE-AL solutions and the complex formation of PE-AL with protein were investigated to clarify the function of this novel lignin derivative. The reduced viscosity of the amphiphile in aqueous solution was low (<0.3 dl/g), and it decreased with increasing concentration in dilute solution. This suggested that the PE-AL in aqueous solution has a structure similar to that of Einstein's sphere and shrinks upon hydrophobic interaction among the structural moieties in AL and the exclusive volume effect. The amphiphilic PE-AL obviously formed a complex with bovine serum albumin (BSA) at 4°C with a reaction time of about 1 week. After complex formation with cellulase for 1 week, the cellulase activity of the resulting complex is significantly enhanced and is preserved after recycling the complex for hydrolysis of cellulosic materials several times.

**Key words** Acetic acid lignin · Amphiphile · Cellulase stabilizer · Complex formation with proteins · Polyethylene glycol diglycidyl ether · Viscosity

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## Introduction

Lignin is an important woody biomass component that can be used as an alternative to fossil resources for the twenty-first century. The nature of lignin preparation depends on the method of isolation or pulping. Most technically prepared lignin is obtained by a kraft pulping process. The resulting kraft lignin is burned in the form of concentrated spent liquor to recover energy and pulping chemicals.[1] Only lignosulfonate obtained by sulfite pulping is utilized as an admixture of cement, dispersant, and so forth.[2] Therefore, technically obtained lignin does not seem to be utilized efficiently despite its potential as a functional polymer. To utilize the woody components in the naturally occurring biopolymers effectively, some urgent problems must be overcome, including (1) development of an efficient, novel method for separating the components of woody tissues without appreciable changes in their chemical structures, and (2) exploration of the utilization of the separated components. We have developed a novel acetic acid pulping process under atmospheric pressure for separating wood components from woody tissues.[3] The lignin preparation (AL) thus obtained shows unique thermal fusion properties[4] and can be transformed into carbon fibers,[5] which are then treated with steam to produce activated carbon fibers.[6] The AL preparation is soluble only in an organic solvent with middle polarity, such as acetic acid, acetone, or pyridine. In this study the AL preparation was converted to a water-soluble derivative (PE-AL) by crosslinking with amphiphilic polyethylene glycol diglycidyl ether (PE).

Lignin preparations and derivatives were reported to have high affinity for proteins.[7] On the basis of this observation, a lignin derivative was used as a carrier for immobilizing the enzyme.[8] From the viewpoint of biomass utilization, it is conceivable that the PE-AL preparation can be applied as a cellulase stabilizer for preserving the hydrolytic activity for a long period (i.e., a carrier for a water-soluble immobilized enzyme system). Cellulase is the enzyme that catalyzes hydrolysis of the $\beta 1 \rightarrow 4$ glycosidic bond of cellulose.

Because the hydrolysis of cellulose catalyzed by cellulase proceeds heterogeneously, the rate of hydrolysis is much slower than that catalyzed by acid, although the reaction temperatures are different. To improve the cellulase activity and to facilitate separation of products from the resulting hydrolysate, several investigations have been conducted on the modification of the substrates[9,10] and immobilization of the enzyme systems.[11] Among the immobilized cellulase systems, the typical system immobilizes cellulase on a solid carrier. This system does not appreciably improve the efficiency of cellulase for hydrolysis at the initial stage because of the low reactivity of immobilized enzymes to water-insoluble substrates (solid–solid phase reaction).[12] On the other hand, the water-soluble immobilized cellulase system showed enhanced cellulase activity in addition to preserving the activity of celullase for a long period.[13,14] Thus, lignin preparation is considered to be one of the promising sources for the carrier system.

In this study, the characteristics of the PE-AL preparation and the complexation of PE-AL with proteins were investigated to clarify the functions of this novel lignin derivative. Moreover, the PE-AL preparation was evaluated as a polymeric carrier for the water-soluble immobilized cellulase system.

## Materials and methods

The AL preparation was obtained by atmospheric acetic acid pulping of birch chips as described previously.[15] Polyethylene glycol diglycidyl ether (PE) with about 13 units of ethylene oxide group [RO—($CH_2$—$CH_2$—O)$_n$—R, where n is 13 and R represents glycidyl] was kindly supplied by Nagase Kasei, Tatsuno, Japan. A 10-g aliquot of AL was dissolved in 100 ml of 1 M aqueous NaOH for 24 h. PE (16 g) was added to the AL solution. The mixture was heated at 70°C for 3 h and allowed to stand at room temperature for 3 days with mechanical stirring. The solution was acidified to pH 4 with acetic acid and then dialyzed against deionized water. After concentration, 15 g of powdery PE-AL was obtained by lyophilization.

The PE-AL preparation was characterized by determining the functional group and infrared (IR) spectroscopy. The ethylene oxide group in PE-AL was determined by the method reported by Morgan.[16] The reactivity of the degradation in the method was corrected based on the reactivity of polyethylene glycol with a molecular mass of 4000. The residual glycidyl group was estimated by the consumption of hydrochloric acid, which reacted with the epoxy group to form chlorohydrin. The concentration of hydrochloric acid was estimated by the chloride ion, which was determined by capillary electrophoresis on a Waters Quanta 4000.

The molecular mass of PE-AL was measured in aqueous and tetrahydrofuran (THF) solutions on size exclusion chromatography (SEC) with a refractive index detector and a column of Shodex 1G-7B (Shodex, Tokyo) that had a void volume of $10^7$ kDa and was calibrated with authentic polystyrene and polyethylene oxide, respectively.

### Viscosity measurement

The PE-AL preparation was fractionated by ultrafiltration with a 30-kDa cutoff membrane. The viscosity of fractionated PE-ALs was measured at 25°C in $CHCl_3$, deionized $H_2O$, and 0.5 M aqueous NaCl solution using an Ubbelohde-type viscometer.

### Complexation with bovine serum albumin

The PE-AL preparation was fractionated by gel filtration with Sephadex G-200 to obtain a high-molecular-mass fraction with small dispersity. The fraction in phosphate buffer solution (1/30 M, pH 7) was mixed with bovine serum albumin (BSA)-buffered solution at 4°C. Aliquots of the mixture were withdrawn every day and were subjected to SEC and circular dichroism. SEC was performed at 37°C using a Shodex GS-520 column with a flow rate of 0.5 ml/min. Similarly, the circular dichroic spectra of the samples with BSA were recorded on a Jasco J-720 model (Tokyo, Japan).

### Complexation with cellulase and evaluation of cellulase activity

The PE-AL preparation in acetate buffer (pH 5.0) was mixed with Meicelase, a commercial cellulase (kindly supplied by Meiji Seika, Tokyo, Japan), for 1 week at 4°C. To examine the stability of the cellulase, the mixture was left for 30 days at room temperature or 40°C. During storage 10 ml of the mixture (cellulase 40 mg; PE-AL 0–0.2 wt% based on the solution) was withdrawn every day and was subjected to hydrolysis of filter paper (Advantec No. 51A, Tokyo, Japan) at 40°C for 24 h. The hydrolysis residue was filtered with a glass filter, washed with a small volume of $H_2O$, and weighed after oven-drying. The weight of hydrolysate was calculated by subtracting the weight of the residue from that of the filter paper tested. Cellulase activity was evaluated by the ratio of the weight of hydrolysate to that of the filter paper tested.

The frequency with which the cellulase could he reused for hydrolysis of cellulosic materials was evaluated by the following procedure. The hydrolysis was first performed under the following conditions: substrate (3 g), cellulase (40 mg), and PE-AL (0.2 wt% based on 300 ml of acetate buffer solution at pH 5.0) at 40°C for 48 h. After removing the hydrolysis residue by filtration, cellulase was separated from the filtrate (which contained the enzyme and hydrolysate) by ultrafiltration with a 10-kDa cutoff membrane made from polysulfone. After washing the cellulase fraction with the buffer solution upon ultrafiltration, cellulase was recovered as a concentrated solution that did not pass the membrane. The recovered cellulase was applied to the second hydrolysis. This procedure was repeated six times.

## Results and discussion

### Chemical structure and solution property of the PE-AL preparation

Lignins have phenolic and aliphatic hydroxyl groups as reactive sites.[17] The acetic acid lignin (AL) had hydroxyl groups in a concentration of 4.74 mmol/g, of which 40% was acetylated during the pulping. After saponification, therefore, the AL preparation was coupled with polyethylene glycol diglycidyl ether (PE), an amphiphilic epoxy compound, to afford a water-soluble lignin derivative. The resulting PE-AL preparation, with only one repeating unit of ethylene oxide, is not soluble in water [RO—($CH_2$—$CH_2$—O)$_n$—R, where n is 1 and R represents glycidyl]. A water-soluble PE-AL preparation can be obtained when the AL preparation is coupled with about 13 repeating units of ethylene oxide in the PE [RO—($CH_2$—$CH_2$—O)$_n$—R, where n is 13 and R represents glycidyl]. A completely water-soluble PE-AL can be prepared when the AL preparation is coupled with PE (where n is 13) with a PE/AL weight ratio of 1.6 at a lignin concentration of 10% (w/v). The resulting PE-AL preparation is soluble in organic solvents, such as methanol, chloroform, and pyridine in addition to water. Thus, the PE-AL preparation is an amphiphile.

When the concentrated lignin solution (33%, w/v) was reacted with PE, AL formed a hydrogel. This phenomenon suggests that PE acts as a crosslink to form a three-dimensional network structure. Accordingly, in low concentration PE coupled AL molecules two-dimensionally in such a manner that an alternating co-polymer was produced from PE and AL.

The chemical structure of the PE-AL preparation was characterized by determining the ethylene oxide moiety and by IR spectroscopy. Figure 1 shows the IR spectra of PE, AL, and their derivatives. In addition to the typical adsorption bands of acetic acid lignin (AL), the IR spectrum of AL shows a distinctive adsorption band corresponding to the carbonyl band of the acetyl group. Because the IR spectrum of PE-AL preparation does not exhibit this band, the coupling of PE to AL must proceed under alkaline conditions after complete hydrolysis of the acetoxyl groups. The spectrum of the PE-AL shows adsorption bands at 1590 and 1510 $cm^{-1}$, corresponding to the aromatic nucleus. In addition, the intense adsorption band at about 2900 $cm^{-1}$ corresponds to the C—H bond of the methylene group. Thus, the PE-AL consists of PE and AL moieties. The $^1$H nuclear magnetic resonance ($^1$H-NMR) spectrum of PE-AL also shows signals for protons of the methylene and aromatic groups (data not shown). Because the methylene signals are much more intensive than signals for protons of other functional groups in the AL moiety, the spectrum cannot be used for quantitative determination of functional groups in the PE-AL. The PE/AL weight ratio in the PE-AL preparation was finally estimated by determining the ethylene oxide unit after acidic degradation,[16] and the residual glycidyl group was quantified by the consumption

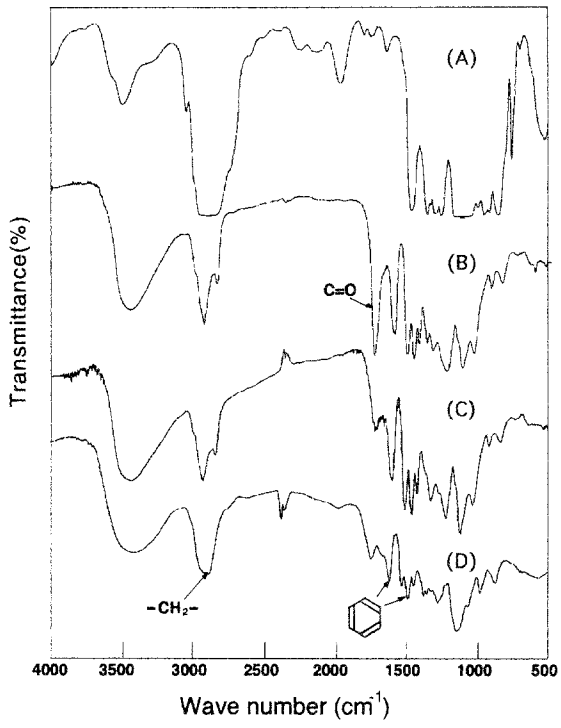


**Fig. 1.** Fourier transform infrared (FT-IR) spectra of polyethylene glycol diglycidyl ether (PE) and acetic acid lignin (AL) preparation. (*A*), PE; (*B*), AL; (*C*), saponified AL; (*D*), PE-AL

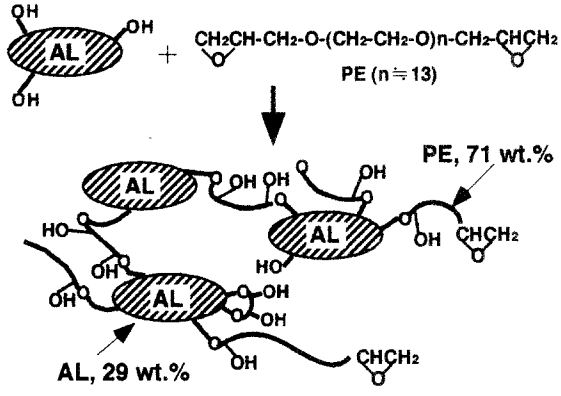


**Fig. 2.** Possible chemical reaction for the formation of PE-AL and structure of PE-AL

of chloride ion upon chlorohydrin formation. The PE content is 71 wt%; and the unreacted, residual glycidyl group is 0.13 mmol/g in the PE-AL. A molecular mass of PE-AL by SEC was a weight average molecular mass (Mw) of 67 × $10^3$ and polydispersity of 1.5 in THF and an Mw of 223 × $10^4$ and polydispersity of 1.3 in aqueous solution after dialysis. The huge Mw in aqueous solution may result from aggregation of PE-AL molecules. It is apparently that PE-AL has a much larger Mw than the original AL (Mw 3800). These results suggest that the AL is converted to a large macromolecule by coupling with the PE. Figure 2 shows the possible chemical reaction for the formation of the PE-AL and its structure.

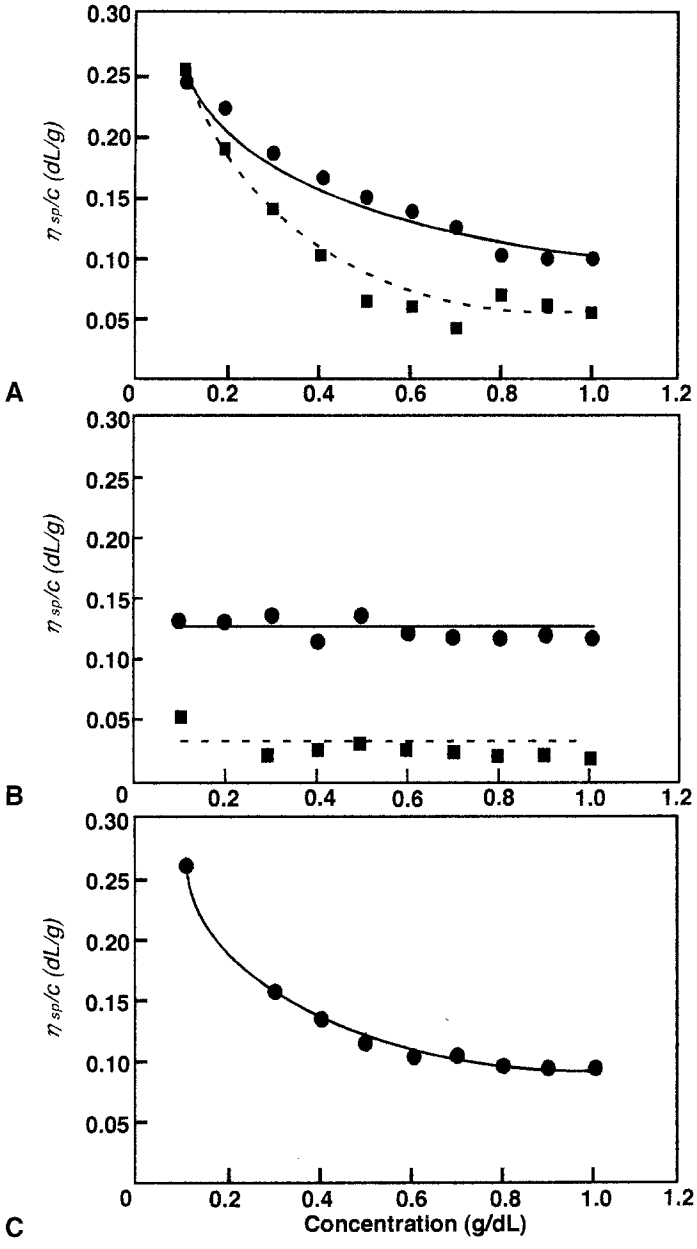


**Fig. 3.** Reduced viscosity of PE-AL (*circles*), molecular weight >30 kDa; *squares*, molecular weight <30 kD in water (**A**), chloroform (**B**), and 0.5 M aqueous sodium chloride solution (**C**) at 25°C

The solution behavior of PE-AL was investigated by viscometry. Figure 3 shows the reduced viscosity ($\eta_{sp}/c$) of PE-AL versus its concentration. Two fractionated PE-AL preparations obtained by ultrafiltration with a 30-kDa cut-off membrane was used for the measurement after dialysis. The plots of reduced viscosity versus concentration of these two fractions in aqueous solution show unexpected curves, which is similar to that of a polyelectrolyte, whereas those in chloroform are straight lines. The plots in 0.5 M sodium chloride solution show similar curves. As expected from the chemical structure, the unique viscometric property of PE-AL can not be attributable to polyelectrolytes. Because $\eta_{sp}/c$ generally corresponds to the hydrodynamic radius of each macromolecule at a certain concentration, the decrease in $\eta_{sp}/c$ with increasing concentration implies a decrease in the molecular size. This fact can be interpreted by the PE-AL molecule shrinking in water because of the hydrophobic interaction among the structural moieties in the AL moiety in addition to the excluded volume effect. Accordingly, $\eta_{sp}/c$ does not change in chloroform, in which the hydrophobic interaction is suppressed.

The $\eta_{sp}/c$ of the PE-AL in both aqueous and chloroform solutions are low (<0.3) in the whole concentration range investigated despite its large molecular mass. This low viscosity leads us to anticipate a solution structure of PE-AL that scarcely interacts with solvent. Hence, the structure is conjectured by an exponent in the Mark-Houwink Sakurada equation. The parameter for PE-AL in the equation is estimated from the intrinsic viscosity and the molecular mass determined by SEC, where we estimate the intrinsic viscosity in aqueous solution to average $\eta_{sp}/c$ at a concentration range of 0.8–1.0 g/dl. The exponents in the aqueous and chloroform solutions are 0.08 and 0.33, respectively. Evidently, the PE-AL has a structure similar to Einstein's rigid sphere in aqueous solution. Therefore, the PE-AL has a unique structure in aqueous solution, forming a dense sphere with increasing concentration because of the hydrophobic interaction.

## Complex formation with protein

The PE-AL is expected to form a complex with protein upon coupling of the residual glycidyl group in addition to physical adsorption. The process of complex formation of PE-AL with BSA was monitored by SEC and circular dichroism. The complex-formation experiment was performed at 4°C to prevent denaturation of BSA at ambient temperature. Figure 4 shows high-performance size exclusion chromatograms of the original PE-AL, BSA, and their mixture. The chromatogram of the mixture just after mixing exhibits two peaks, corresponding to PE-AL and BSA. The chromatogram almost did not change for 5 days. After 6 days the latter peak, corresponding to the BSA, decreased day by day. According to the circular dichroic spectra (Fig. 5), a negative cotton effect attributable to the $\alpha$-helix structure of the BSA was reduced after 5 days. The change in the secondary structure of the BSA is evidence of complex formation. These results suggested that the PE-AL obviously forms a complex with the protein at 4°C with a reaction time of 1 week.

## Complex formation with cellulase and hydrolytic activity of cellulase

The PE-AL was mixed with cellulase in acetate buffer solution (pH 5.0) at 4°C for 1 week with gentle stirring to form a tight complex. The cellulase activity of the complex was evaluated by the hydrolysis ratio of several cellulosic materials, as shown in Table 1, where the hydrolysate obtained by the complex hydrolysis for 1 day could not be analyzed because of shortage of the hydrolysate for the analysis.

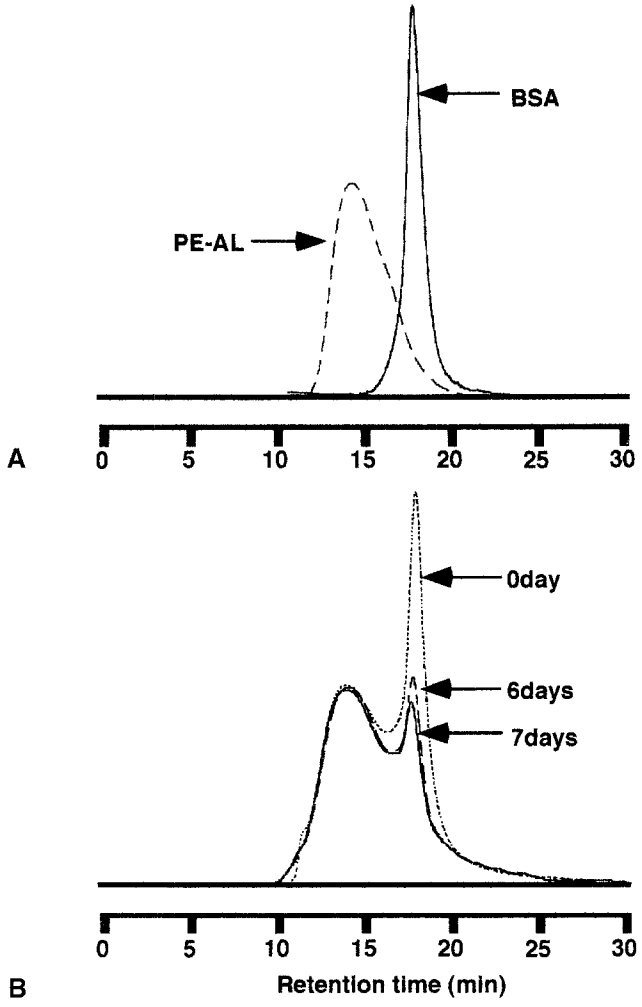


**Fig. 4.** Size exclusion chromatograms of PE-AL and bovine serum albumin (*BSA*) (**A**), and their mixture (**B**)

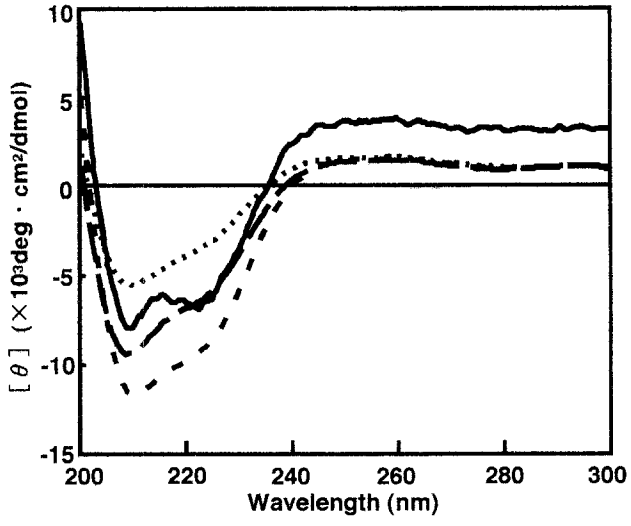


**Fig. 5.** Circular dichroic diagrams of BSA and PE-AL complex. *Solid line*, BSA; *heavy broken line*, complex at 1 day; *light broken line*, complex at 3 days; *dotted line*, complex at 5 days

**Table 1.** Enzymatic hydrolysis of cellulosic materials by cellulase and PE-AL–cellulase complex

| Enzyme and substrate | Hydrolysis ratio (%) | | Sugars produced (%) | | | |
|---|---|---|---|---|---|---|
| | | | 24 h | | 48 h | |
| | 24 h | 48 h | Glc | CB | Glc | CB |
| Cellulase | | | | | | |
| F-P | 54.1 | 77.9 | 91.7 | 8.3 | 100 | 0 |
| P-P | 38.4 | 63.0 | 86.7 | 13.3 | 100 | 0 |
| D-P | 45.2 | 52.6 | 84.0 | 16.0 | 99.9 | 0 |
| Complex | | | | | | |
| F-P | 55.7 | 80.0 | – | – | 100 | 0 |
| P-P | 41.2 | 75.2 | – | – | 100 | 0 |
| D-P | 40.5 | 59.3 | – | – | 99.9 | 0 |

F-P, filter paper; P-P, PHA pulp; D-P, deinked pulp; Glc, glucose; CB, cellobiose; 24 h and 48 h, hydrolysis time
Hydrolysis condition: substrate 500 mg; cellulase 40 mg; PE-AL 0.2 wt% based on the buffer solution; buffer solution 50 ml of acetate buffer (pH 5.0)

**Table 2.** Enzymatic hydrolysis of filter paper by cellulase at 40°C for 24 h

| Concentration (%) | Hydrolysis ratio (%) |
|---|---|
| PE | |
| 0 | 54.1 |
| 0.01 | 54.1 |
| 0.05 | 52.0 |
| 0.10 | 45.1 |
| 0.50 | 33.6 |
| PE-AL (0.2%) | 55.7 |

Filter paper is ADVANTEC No. 51A (Tokyo Roshi Kaisha, Ltd.)
Experimental conditions: substrate 500 mg; cellulase 40 mg; acetate buffer (pH 5.0) 50 ml; time 24 h; temperature 40°C

Compared to the activity of intact cellulase, the cellulase activities of the complex do not diminish during complex formation but are comparable to or higher than those of the cellulase. These results are rare cases for immobilization of enzyme.[18] On the other hand, the cellulase activity is reduced by addition of only the PE, as shown in Table 2. It is caused by denaturation of cellulase upon coupling of the enzyme with the PE. Thus, coupling the enzyme with PE negatively influences cellulase activity.

The effect of PE-AL on cellulase storage was examined at 4°C, room temperature, and 40°C. As shown in Fig. 6, when filter paper was used as substrate the hydrolytic activity of intact cellulase decreased to 95% at room temperature and to 87% at 40°C after 30 days. In contrast, the activities of the complex with a cellulase/PE-AL ratio of 2:1 by weight (0.2 wt%) are 99% and 90% under the same storage conditions, respectively. Thus, the PE-AL is found to preserve the hydrolytic activity of cellulase in aqueous solution for a long time.

The effect of PE-AL on cellulase recycling was investigated in a continuous multistage saccharification process of cellulosic materials using cellulase as catalyst. Ultrafiltration was used to separate the cellulase employed from the hydrolysates in the reaction medium.[19] The hydrolyses were

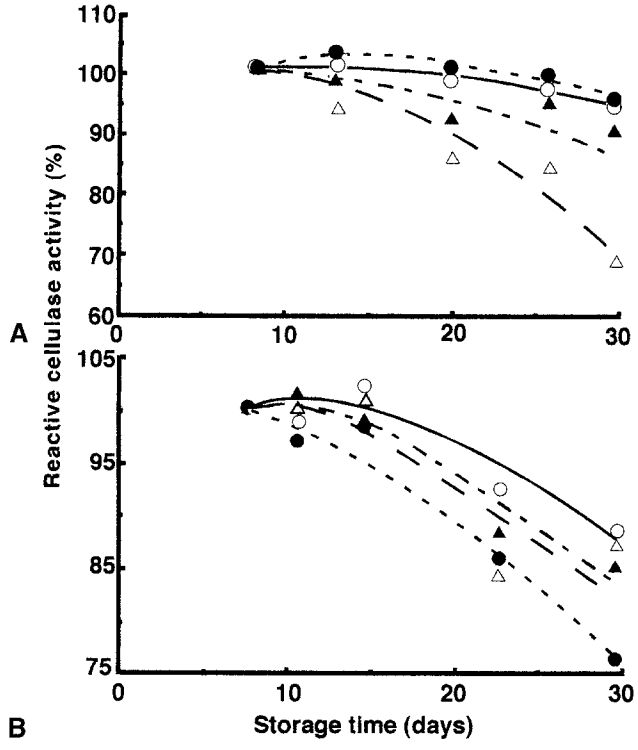


**Fig. 6.** Cellulase activity of PE-AL – cellulase complex during a long storage time at room temperature (**A**) and 40°C (**B**). Hydrolysis conditions: filter paper 0.5 g; cellulase 40 mg. PE-AL concentrations: *filled triangles*, 0 wt%; *filled circles*, 0.02 wt%; *open circles*, 0.2 wt%; and *open triangles*, 2.0 wt% on the buffer solution

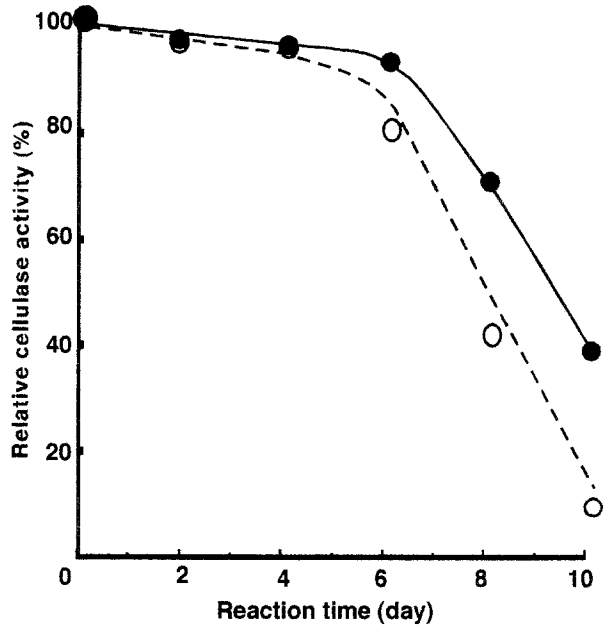


**Fig. 7.** Multistage recycling process for enzymatic hydrolysis of filter paper by cellulase and PE-AL – cellulase complex using ultrafiltration. Hydrolysis conditions: substrate 3 g; cellulase 240 mg; PE-AL 0 wt% (*open circles*) and 0.2 wt% (*filled circles*) based on 300 ml of the buffer solution

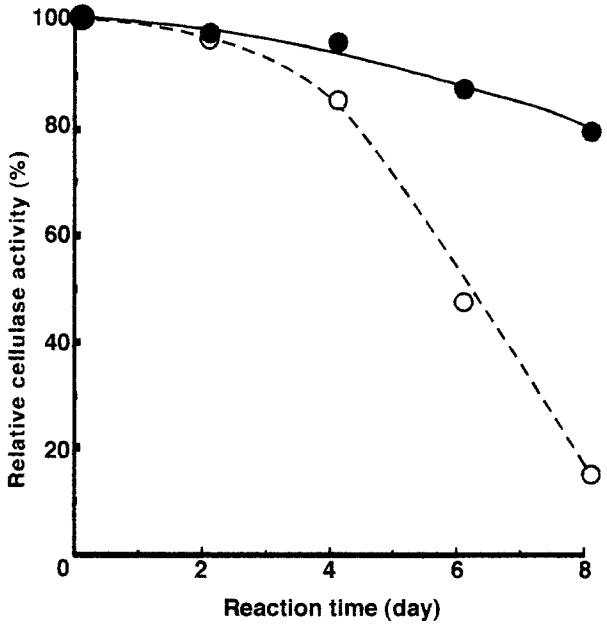


**Fig. 8.** Multistage recycling process for enzymatic hydrolysis of PHA-P (todo fir) by cellulase and PE-AL – cellulase complex using ultrafiltration. Hydrolysis conditions: substrate 3 g; cellulase 240 mg; PE-AL 0 wt% (*open circles*) and 0.2 wt% (*filled circles*) based on 300 ml of the buffer solution

conducted for 48 h; the substrates were filter paper and polyhydric alcohol (PHA) pulp, which is a new type of organosolv pulp. This process requires a large volume of buffer solution to separate the cellulose employed by ultrafiltration. To keep the concentration of PE-AL the same as during the previous stage in the recycling experiments, the PE-AL was used in large quantity; the cellulase/PE-AL weight ratio was 0.4. Figures 7 and 8 show the relative cellulase activity, expressed as a percentage based on the initial hydrolytic (cellulase) activity as aforementioned. The initial activity of the PE-AL–cellulase complex is higher than that of intact cellulase. When filter paper is used as substrate, both residual activities are the same until the third recycling, and then a difference is observed. The residual activities of the complex are 70% at the fifth recycling and 40% at the sixth recycling, and those of cellulase are 40% and 15%, respectively. When PHA pulp is used as substrate, the residual hydrolytic activity of the complex is 80% of the initial activity at the fifth recycling, whereas that of cellulase is only 15% at the same stage. For all enzymatic hydrolyses, the resulting hydrolysates are comprised of only one product (i.e., glucose). This result suggests that cellulosic materials were converted to glucose efficiently without reducing $\beta$-glucosidase activity. Therefore, PE-AL is a useful lignin derivative for preserving cellulase activity when recycling during the hydrolysis of cellulosic materials.

We propose two reasons for PE-AL being effective in preserving cellulase activity. PE-AL facilitates the release of cellulase from substrate,[20] as PE-AL has a weak surface activity compared to polyethylene glycol. Figure 9 shows evidence of weak surface activity of PE-AL, which is measured by the solution drop method. Furthermore, PE-AL contributes to stabilization of the cellulase structure in solution because of the hydrophilic and hydrophobic interac-

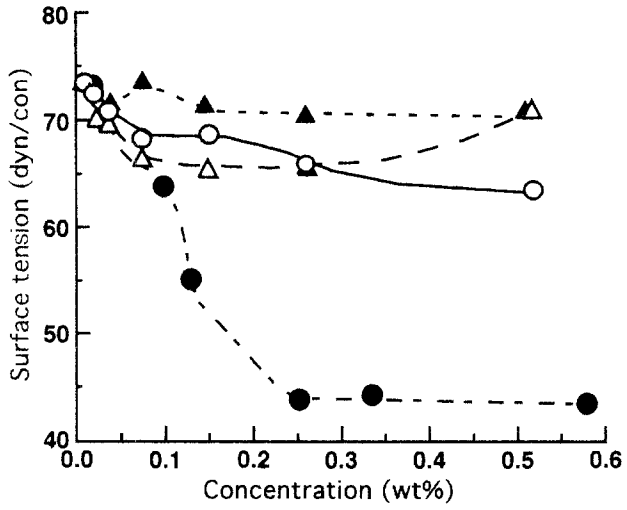


**Fig. 9.** Surface tension of sodium dodecyl sulfate (SDS), polyethylene glycol (PEG), and PE-AL. *Filled circles*, SDS; *filled triangles*, PEG 600 (MS = 600); *open triangles*, PEG 20000 (MS = 20000); *open circles*, PE-AL

tion: PE-AL has a hydrophilic domain of the PE and hydrophobic domain of the AL. In addition, because PE-AL seems to interact with proteases in crude Meicelase, which causes degradation of cellulase, the effect on cellulase activity is being investigated. Consequently, PE-AL is an excellent, water-soluble polymeric carrier for immobilization of cellulase to preserve the hydrolytic activity for a long period.

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