Modification of Cellulase by Synthesized Copolymer with Polyethylene Oxide and Maleic Acid Anhydride

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ABSTRACT: Copolymer containing functional groups such as polyethylene oxide (PEO) and maleic acid anhydride (MA) was synthesized to modify cellulase. MA was attached to the PEO allyl ester, which was the product formed by the reaction between PEO allyl alcohol and lauric acid. The number of ethylene oxide (EO) units in one PEO chain was varied from 10 to 40, and MA formed the chemical bond with the amino acid groups of the cellulase for the modification reaction. When cellulase was modified with synthesized copolymer, activity of the modified cellulase decreased slightly as the degree of modification increased. The modified enzyme showed high remaining activity regardless of a high degree of modification. At the maximum modification degree of 52%, the modified cellulase activity retained more than 65% of the unmodified native cellulase. Modified cellulase retained higher reactivity than native cellulase in an organic solvent and at various pH values. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 77: 368–373, 2000

Key words: poly(oxyethylene); maleic acid anhydride; modification; stability of enzyme activity; degree of modification

INTRODUCTION

Environmental problems are becoming a major concern for everyone on earth. Recycling useful waste is one solution that can alleviate some of these problems. For instance, wastepapers can be collected and treated for reuse, and waste cellulosic materials can be transformed into useful chemicals by enzymatic conversion. For enzymatic conversion, the high cost and instability of the cellulolytic enzyme have been the major problems associated with this process.[1–3] Park et al.[4] showed that nonionic surfactant increased the rate and extent of saccharification of the substrate and thereby improved the recovery of the enzyme. In that case, the surfactant created a hydrophilic environment that played an important role in the control of adsorption and desorption processes of cellulase on the cellulose surface and enhanced saccharification of the substrate. Inada and Nishio[5–6] have modified enzymes such as lipase, catalase, chymotripsin, and peroxidase with a copolymer of monomethoxy polyethylene glycol and cyanuric chloride. The modified enzymes were soluble in organic solvents and showed enzymatic activity in the solvents. According to the above information, it may be expected that combining cellulase with a synthetic polymer such as a PEO derivative will show additional properties of the nonionic surfactant and synthetic polymer.

In this study new copolymers containing functional groups such as MA and PEO allyl ester were synthesized, and they were used to modify the amino acid groups of the cellulase. The remaining activity of modified cellulase was determined, and the stability of the modified cellulase in an organic solvent and in various pH environments were studied.

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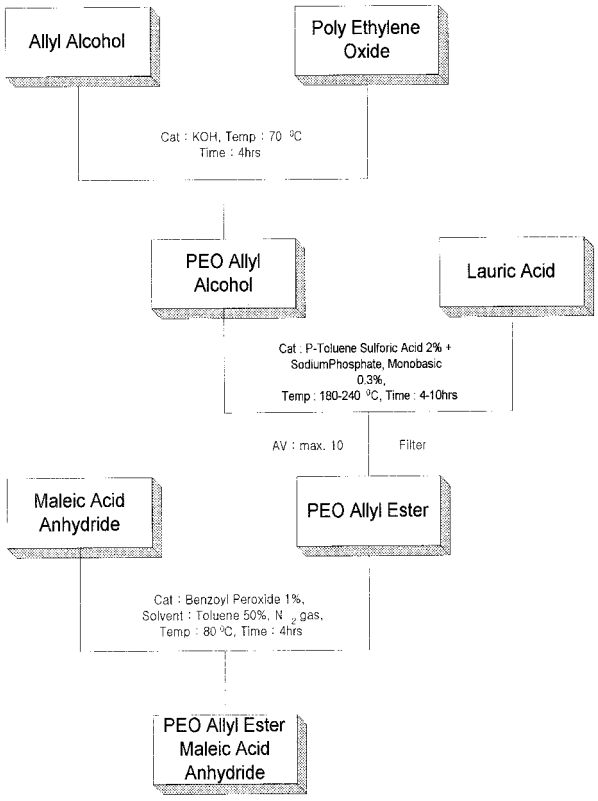

Figure 1 Schematics of the copolymer synthesis.

EXPERIMENTAL

Materials

Y-NC, a crude cellulase from *Aspergillus Niger,* was purchased from Yakult Co., Japan. Filter paper hydrolase (FPase) activity was analyzed by using filter paper FP-5C (Toyo Roshi Ltd., Japan) as a standard substrate. FPase activity in the cellulase was 0.19 unit/mg, with the protein at pH 5.2 and 50°C, which was assayed as reported by Mandels and coworkers.[7] Activity of 1 unit is defined as 1 μmol of glucose equivalent released from the substrate per minute. A unit of activity therefore is defined by the amount of enzymes, which produced 1.0 μmol of reducing sugar from the substrate per minute. Reducing sugar was determined by the dinitrosalicylic acid (DNA) method with glucose as a standard.[8] PEO with different chain lengths was obtained from Korea Polyol Co., Korea. The rest of the chemicals used to synthesize the copolymer were all ACE grade and were obtained from Sigma-Aldrich, Korea.

Experiments

Synthesis of Copolymer

Copolymer, which consists of polyethylene oxide (PEO) and maleic acid anhydride (MA), was used as a modifier, and it was synthesized as follows (Fig. 1). First, allyl alcohol was combined with PEO at 70°C for 4 h inside an autoclave with KOH as a catalyst. The number of EO units in one PEO chain was varied from 10 to 40 in order to see the effect of the EO length. Then, in an esterification reaction, PEO allyl alcohol was combined with lauric acid to form an ester. The mixture of PEO allyl alcohol and lauric acid was allowed to react 4-10 h under 2% *p*-toluene sulfonic acid and 0.3% sodium phosphate catalyst, while the temperature was increased from 180 to 240°C. The conversion was calculated by the acid value, which was defined as the number of milligrams of KOH to neutralize 1 g of the acid sample. The acid value can be found by acid–base titration with a phenolphthalein indicator. Finally, MA was added to the ester to form the desired copolymer compound. It is thought that PEO makes the copolymer into a hydrophilic environment in water, while MA gives the functional group necessary to bond with the amino acid groups of the enzyme. The reaction mechanisms for the synthesis of the copolymer are also shown in Figure 2. Structural analysis was carried out by infrared spectrometry

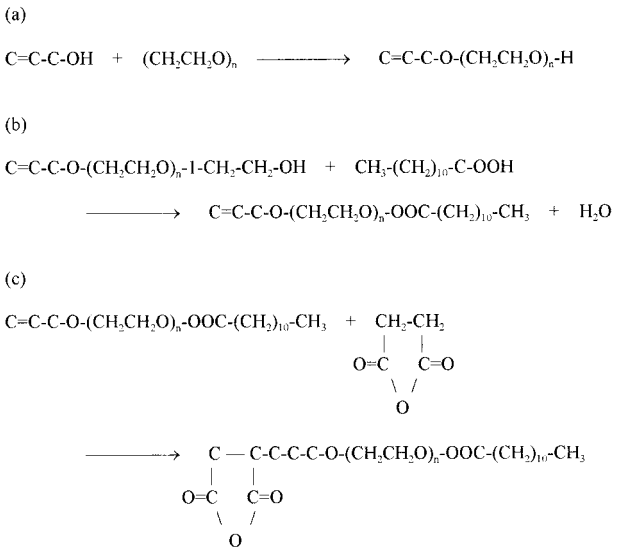

Figure 2 Mechanism of polymer synthesis: (a) PEO is added to allyl alcohol; (b) esterification reaction between PEO allyl alcohol and lauric acid; and (c) addition of MA in PEO allyl ester.

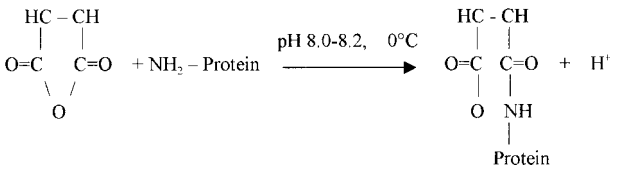

Figure 3 Reaction of cellulase modification with maleic acid anhydride.

(IR, JASCO IR-700) and Fourier transform nuclear magnetic resonance (FTNMR, Bruker 250 spectrometer).

Modification of Cellulase with Synthesized Copolymer

Synthesized copolymer was combined with cellulase by maleylation to form modified cellulase,[9] and its reaction scheme is shown in Figure 3. Maleylation is one of the chemical modifications of protein with MA. This reaction occurred effectively under the condition of pH 8.0 and low temperature. However, as the reaction proceeded, pH decreased due to the production of carboxylic acid, and it was thus necessary to control the pH value with a base. In the case of modification with copolymer, cellulase reacted easily with the MA group of the copolymer. Modification of cellulase with the copolymer modifier was carried out as follows: Copolymer was added stepwise to the cellulase solution, and the mixture was slowly stirred at 4°C under pH 8.0–8.2, which was controlled with 0.2*M* NaOH. The degree of modification (*DM*) of cellulase with modifiers was defined as follows:

$$DM = 1 - \frac{\text{Unmodified } NH_2 \text{ of modified cellulase}}{\text{Total } NH_2 \text{ of native cellulase}}$$

The amino groups of the cellulase were determined using the trinitrobenzene sulfonic acid (TNBS) method.[10] The *DM* was varied by changing the weight ratio of copolymer to cellulase over the range of 0.1–6.0 (w/w).

Stability of Modified Cellulase Against Organic Solvent and pH Variation

The stability of modified cellulase in organic solvent was studied. Water-soluble solvents such as ethanol and acetone were used in the recovery of the enzyme, so a water-insoluble organic solvent, benzene, was used in this experiment. Solutions containing 20% benzene and the modified cellulase solution were mixed for 20–60 min at 50°C. After conservation, the remaining activities were determined. The stability of modified cellulase in various pH solutions was studied. Modified cellulase was incubated under pH 5.5 and 8.5 buffer solutions for 12, 24, and 36 h at 50°C. The reaction pH was chosen for the enzymatic application of the pulp/paper industry. Both native and modified cellulase can be applied to wastepaper reprocessing. And wastepaper slurries had a pH of 8.5 after the pulping process when no chemicals were added. The remaining activities were measured as mentioned above.

RESULTS AND DISCUSSION

Characteristics of Synthesized Copolymer

Copolymer was synthesized, and its reaction path is shown in Figure 3. First, polyethylene oxide (PEO) was added to the allyl alcohol. PEO was added to oxygen in the OH group of allyl alcohol. Then, PEO allyl alcohol underwent an esterification reaction with lauric acid. This step was necessary to prevent a side reaction for the MA addition. The OH group in the allyl alcohol was neutralized to produce water (H_2O) with H from lauric acid, while the carboxylate group of the lauric acid underwent esterification. Finally, MA was added to the PEO allyl ester to make the final product. Before the final reaction, both MA and the allyl ester had a carbon double bond. The carbon double bond present in the MA was broken down into a carbon single bond and was added to the double bond of the PEO allyl ester by breaking it into a carbon single bond.

PEO was added to the allyl alcohol at 70°C and mixed for 4 h inside an autoclave with KOH as a catalyst in the first step of the synthesis. The characteristics of PEO allyl alcohol are shown in Table I. The product was in a liquid state when the PEO with 10 EO units was used, while the products were paste forms when more than 10 EO units were used. The hydroxyl value decreased as the number of EO units of PEO increased. The hydroxyl value is the amount of KOH needed to neutralize H^+ from allyl alcohol. Allyl alcohol loses a hydrogen ion when it reacts with a PEO chain. PEO allyl alcohol was transformed into its ester form in order to combine with MA without opening its ring structure. PEO allyl alcohol was combined with lauric acid to form an ester, and the conversion could be calculated by measuring

Table I Characteristics of PEO Allyl Alcohol

	Allyl Alcohol (10)	Allyl Alcohol (20)	Allyl Alcohol (30)	Allyl Alcohol (40)
EO mol number	11.0	21.9	30.8	39.6
Molecular weight	544.8	1020.6	1413.8	1798.1
Hydroxyl value	103.0	55.0	39.7	31.2
Color	APHA20	Gardner1	Gardner1	Gardner1

the acid value of the ester product. The case of the PEO with 10 EO units is shown in Figure 4. As the reaction temperature increased, consumption of acid by the reaction with alcohol increased, and thus acid value of the reactant decreased while conversion increased. Acid value and conversion were 3.3 and 95.8%, respectively, at 240°°C. Allyl esters of different numbers of EO units were manufactured and are shown in Table II. As the EO number increased, the saponification value and the temperature that completes the reaction decreased. Conversion values for each EO at unit lengths of 10, 20, 30, and 40 were 95.8, 93.2, 87.1, and 85.3%, respectively. PEO allyl ester was combined with MA, and the product was analyzed with IR and FTNMR for its structure. The IR showed functional groups such as ester (1775, 1745, and 1054 cm^{-1}), MA (1848, 1775, and 1745 cm^{-1}), ethylene oxide (1848, 1775, 1745, and 1054 cm^{-1}), and benzene (3082, 1465, 948, 888, 848, and 722 cm^{-1}), and these coincided with the results from FTNMR. From the FTNMR analysis, 75% of the carbon double bonds in MA (7.07–7.27, 7.26 ppm) were converted into the carbon single bonds(2.32 ppm). We thought that the synthesis of the copolymer with the MA functional group was successfully synthesized with 75% completion.

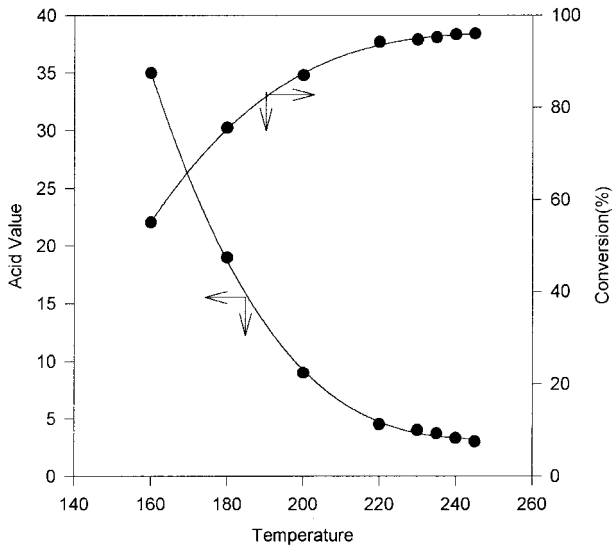

Figure 4 Acid value and conversion of allyl ester according to reaction temperature.

Modified Cellulase with Synthesized Copolymer

The degree of modification (*DM*) and the relative FPase activity according to the weight ratio are shown in Figure 5. Relative FPase activity is defined as the ratio of the FPase activity of modified cellulase to that of native cellulase. As the weight ratio of a copolymer increased, the DM increased and the relative activity decreased.[4] There was scarcely any change for DM and relative FPase activity for a weight ratio more than 2. With a different EO number, the relative activity increased as the number of EO units increased, but DM decreased. More than 65% of relative activity was retained at the highest degree of modification (52%). It is thought that PEO chains can be chemically attached to cellulase without great loss of

Table II Characteristics of PEO Allyl Ester

	Allyl Ester (10)	Allyl Ester (20)	Allyl Ester (30)	Allyl Ester (40)
EO mol number	11.0	21.9	30.8	39.6
Acid value	3.3	3.3	4.6	4.4
Sopanification value	75.1	51.0	37.9	29.7
Conversion[a] (%)	95.8	93.2	87.1	85.3

[a] Conversion = [(Initial acid value − Final acid value) × 100]/Initial acid value.

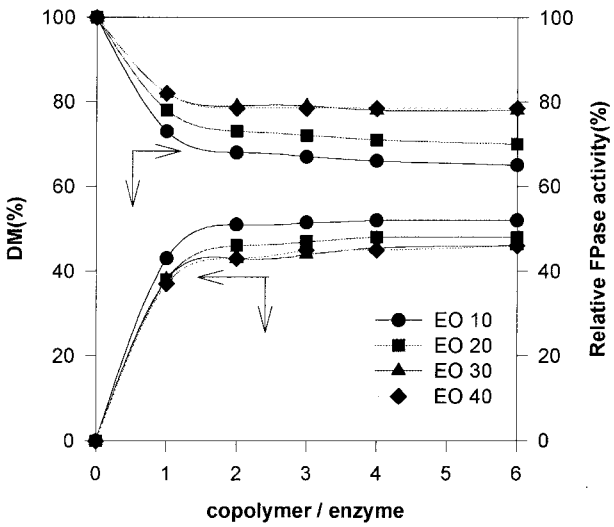

Figure 5 Degree of modification and relative FPase activity of modified cellulase according to weight ratio of copolymer to cellulase.

activity, and modified cellulase will have additional properties from the supporting materials.

Synthesized copolymer was tested for its compatibility with cellulase. Variation of molecular weight was analyzed with gel permeation chromatography (GPC) chromatogram (Fig. 6). The molecular weights of the compounds were extrapolated to the standard curve. Free cellulase showed peaks at 13.4 and 15.5 min, while the peaks ranged from 17 to 20 min for the copolymer

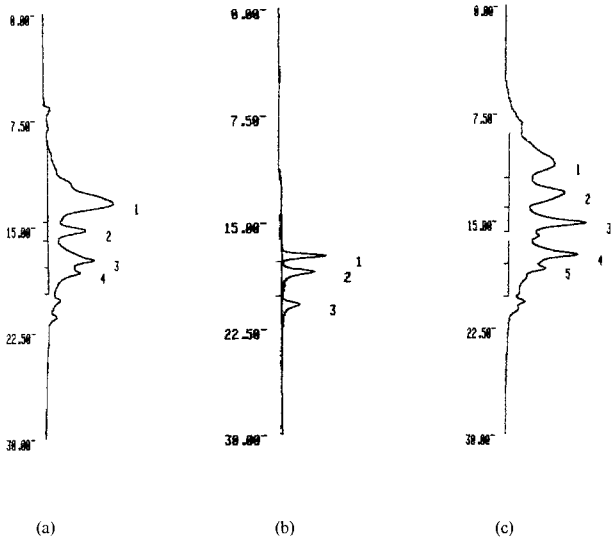

Figure 6 GPC chromatogram of modified cellulase: (a) Y-NC, free cellulase; (b) synthesized copolymer with PEO (10 mol); and (c) modified cellulase.

Table III Stability of Enzyme Solution in Organic Solvent with Benzene

Copolymer/Cellulase (g/g)	Remaining Relative Activity According to Incubation Time			
	0 min	20 min	40 min	60 min
0	100	82.5	39.2	16.9
0.5	73.0	87.4	73.3	62.7
1	68.0	77.6	69.8	61.7
2	67.1	73.4	68.1	61.8
4	65.0	76.4	71.5	65.4

with EO of 10 units. Peaks appeared from 10 to 11 min for the modified cellulase. Thus, it is possible to modify the cellulase with synthesized copolymer. The degree of polymerization is between 1 and 2 based on comparing it to the standard curve.

Stability of Enzyme Solution in Organic Solvent and pH Variation

The stability of enzymes, which is expressed by the relative remaining activity, was studied in organic solvent with benzene and is shown in Table III. Relative remaining activity is defined by the ratio of remaining activity to initial FPase activity. Copolymer with EO 10 units in length was used to modify the cellulase, and the solution mixture of the enzyme solution and benzene was placed inside a water shaker at 50°C at 80 rpm. Native cellulase was rapidly deactivated with incubation time, while the activity of modified cellulase slowly deteriorated and showed higher remaining activity than the native one.[4] Even when 0.5 g of synthesized copolymer was used to modify 1 g of cellulase, it retained more than 62% of the initial activity, while native cellulase retained only 17% of the initial activity. It can be said that the PEO chain of the modifier (copolymer) provides a buffering action against denaturation of cellulase activity.

When the variation in pH was studied (data not shown), native cellulase showed higher activity for a pH in the range of 4 to 6. Around pH 6.5, the activity of both native and modified cellulase was the same, and from that point on, modified cellulase showed better activity than the native cellulase. Native and modified cellulase were different weight ratios were incubated at pH 8.5 and 50°C (Table IV). That with a pH of 8.5 was chosen because the cellulase would have a future appli-

Table IV Stability of Enzyme at pH 8.5

	Remaining Relative Activity According to Incubation Time			
Copolymer/Cellulase (g/g)	0 h	12 h	24 h	36 h
0	100	54.4	34.7	18.9
0.5	73.0	43.6	41.5	40.1
1	68.0	57.1	53.1	50.5
2	67.1	44.4	40.6	39.0
4	65.0	47.5	40.3	39.4

cation in the pulp/paper industry. Most the wastepaper has a pH of 8.5 without any chemical addition when it is disintegrated, and modified cellulase with synthesized copolymer can be applied to wastepaper reprocessing. Thus, pH stability was analyzed as a pH of 8.5. Free cellulase showed a great decrease in activity according to incubation time. After a 36-h incubation, relative remaining activity was only 16.9%. Modified cellulase also lost its activity, but it retained higher relative remaining activity than the native one. These results suggest that the modifier of the cellulase surface created a buffering action against denaturation by the organic solvent and by the alkaline environment.[11]

CONCLUSIONS

Modification of cellulase by synthesized copolymer with polyethylene oxide and maleic acid anhydride was studied. A PEO allyl ester was combined chemically with maleic acid anhydride (MA), and the synthesized copolymer was confirmed by IR and FTNMR. A GPC chromatogram also confirmed that the synthesized copolymer could be combined with cellulase.

As the weight ratio of a copolymer increased, the degree of modification (DM) increased and the relative activity decreased. The DM ranged from 46 to 52%, while relative activity ranged from 65 to 80% for the modified cellulase.

Modified cellulase also displayed a high stability in a water-insoluble organic solvent, benzene, and in an alkaline solution. It can be said that the PEO chain of the modifier (copolymer) provided a buffering action against denaturation of cellulase activity.

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