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CHARACTERISTICS OF CELLULASE MODIFIED WITH AMPHIPHILIC COPOLYMER IN ORGANIC SOLVENT

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Abstract—Characteristics of modified cellulases in organic solvents were studied. Cellulases modified with amphiphilic copolymer of polyoxyalkylene (POA)-derivative and maleic acid anhydride (MAA). Amino groups of the cellulase molecule were easily coupled with the MAA functional group of the copolymer. At the maximum degree of modification (DM) of 55%, the activity of modified cellulase retained more than 80% of the unmodified native cellulase activity. The modified cellulase using AKM-1511 with DM greater than 40% was found to be more than 90% soluble in aqueous solution of acetone and ethanol, leaving the native cellulase and impurities in the fermentation broth with the residue. Modified cellulase showed excellent stability against water-insoluble solvent. Moreover, cellulase modified with hydrophobic copolymer, which consists of ethylene oxide (EO) and propylene oxide (PO), could be dissolved in these solvents.

Key words: Modified Cellulase, Amphiphilic Copolymer, Stability, Solubility in Organic Solvent

INTRODUCTION

Enzymatic conversion of waste cellulosic materials to useful chemicals is a very promising process because cellulose is the most abundant renewable resource. However, high cost and consumption of cellulolytic enzymes have been one of the major problems associated with this process [Clanet et al., 1988; Rivers and Emert, 1988]. For these reasons, cellulase recovery after saccharification has been proposed. Sinitsyn [1983] have reported that cellulase which was adsorbed onto lignin residue was recovered after saccharification. Tjerneld et al. [1985] studied cellulase saccharification in two-phase system and separated both products and cellulase by the combination of membranes.

On the other hand, Some researchers have used a nonionic surfactant during enzymatic hydrolysis in order to increase the rate and extent of saccharification and thereby improve the recovery of enzymes [Castanon and Wilke, 1981; Ooshima et al., 1986; Park et al., 1992]. In the above case the surfactant, which produces a hydrophilic environment, played an important role in the control of adsorption and desorption processes of cellulase on the cellulose surface, and thereby enhanced saccharification of substrate. Inada et al. [1986] and Nishio et al. [1987] have modified enzymes such as lipase, catalase, chymotripsin and peroxidase with 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 informations, it was expected that combining cellulases with synthetic polymer such as polyethylene glycol (PEG) derivative would show additional properties of similar to those demonstrated by nonionic surfactant and/or synthetic polymer.

In our previous work [Kajiuchi and Park, 1992; Kajiuch et al., 1993], cellulases were modified by synthetic copolymers, such as α-allyl-ω-methoxypolyoxyalkylene and maleic acid anhydride (MAA). The modified cellulases displayed a high stability of activity against temperature and pH and showed 30% greater conversion of filter paper as substrate at 90 hours than the native cellulases. It was also expected that the cellulase modification with the copolymer, which could be directly used as a polymer of the aqueous two-phase system was useful to the partition cellulase into the copolymer phase. Modified cellulase was effectively separated by the reactive two-phase system using the amphiphilic copolymers [Moon et al., 1993].

In this study, cellulase modification with new amphiphilic copolymer was carried out to improve the enzyme stability and solubility in organic solvents and to recover cellulase from the native cellulase and impurities.

CONCEPT OF MODIFIED CELLULASE IN SACCHARIFICATION PROCESS

There are some important points that have to be considered during the cellulose enzymatic hydrolysis process; 1. Production of highly active cellulase, 2. Purification of cellulase from the culture broth, 3. Stability of activity against reaction environment such as temperature, pH etc., 4. High conversion of substrate during saccharification, 5. Recovery of free cellulase from product for reuse. To solve the problems mentioned above, cellulase was modified with synthetic copolymer, such as POA derivative and MAA. In purification, there are two characteristics of cellulase modification for effective separation methods; the first is the reactive aqueous two-phase partition, which can concentrate modified cellulase to modifier copolymer phase, and the second is the solubility in aqueous solution of acetone and ethyl alcohol with sedimentation removal of the impurities. In saccharification process, the modified cellulase displayed high remaining activity in spite of modification and high stability of activity against temperature and pH because of the buffering effect of POA chains. Cellulase adsorption onto cellulose could also be controlled by modification. From these results, modified cellulase showed great conversion of substrate than the native cellulase. In addition, modified cellulase showed stability and solubility in water-insoluble organic sol-

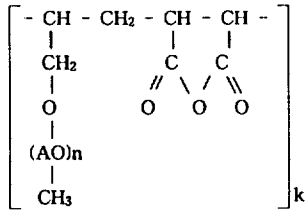
Table 1. Characteristics of cellulase

Origin	Product	Protein[1] [%]	Reducing sugar[2] [%]	FPase activity[3]
T. viride	Onozuka R-10	29.7	37.5	0.23
	Onozuka 3S	8.9	60.0	0.44
A. niger	Y-NC	21.1	32.4	0.19
A. cellulolyticus	Acremonium	34.6	26.6	0.40

1) Total protein of cellulase was determined by the Lowry method with vobine serum albumin as a standard.
2) Reducing sugar was determined by the dinitrosalicilic acid (DNS) method.
3) Specific FPase activity (Unit/mg, protein) was assayed at pH 5.2 and 50℃.

Table. 2. Characteristics of synthetic copolymers

	n	k	MW	EO/AO(%)*
AKM-0531	10	30	18,000	100
AKM-1015	19	14	14,000	100
AKM-1511	33	10	16,000	100
AKM-2010	41	11	21,000	100
ADM-1511	26	11	18,000	30
ACM-1611	29	10	18,000	20



*: EO/AO means ethylene oxide concentration in POA chain.
Abbreviations: AO, alkylene oxide; EO, ethylene oxide

vent and could be recovered from the hydrolytic residue using solvent extraction based on solubilities. From this, it was expected that cellulase modification using an amphiphilic copolymer could be effective procedure to the overall system of cellulose saccharification.

EXPERIMENTAL

1. Material

As listed in Table 1, four types of cellulases produced were used in this study. Onozuka R-10, Onzuka 3S and Y-NC were purchased from Yakult Co., Japan. Acremonium were kindly provided from WEIJI LTD., Japan. The protein contents varied with the types of cellulase and the contents of impurities (i.e., reducing sugar existing in cellulase power). The enzyme solution used as a model for fermentation broth or hydrolysis residue. Activity of the cellulase was represented by FPase activity, which was assayed as reported by Mandels et al. [1976] with FP-5C (Toyo Roshi Ltd., Japan). Reducing sugar was determined by the dinitrosalicilic acid (DNS) method [Miller, 1959] with glucose as a standard. A unit of activity was defined by the amount of enzyme which produced 1.0 μmole of reducing sugar from the substrate per minute.

The species of POA derivative copolymer are listed in Table 2. The alternating copolymer of POA derivative and MAA (Nippon Oil & Fats Co., Japan) are characterized by k and n, where k

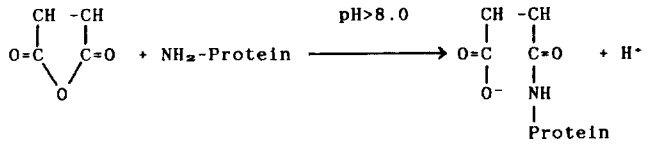


Fig. 1. Reaction of cellulase modification with maleic acid anhydride.

indicates the degree of copolymerization, and n indicates the number of alkylene oxide (AO) units in one POA chain. As k increased, the number of MAA functional group increased. The POA chain consists of EO and PO. The relative hydrophobicity of copolymer increased as the value of EO/AO decreased. Moreover, reactivity of MAA groups with amino acid group of cellulase could be expected.

2. Cellulase Modification

Maleylation is one of the chemical modification of protein with MAA [Butler et al., 1969], and its reaction scheme is shown in Fig. 1. Amino groups of the cellulase molecule were covalently coupled with the MAA 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 pH value with a base. In the case of modification with the copolymer, while the same method was used to treat cellulase, cellulase reacted easily with the MAA function of the copolymer. This reaction occurred effectively under the condition of 4℃ and pH of 8.0-8.2, which was controlled with 0.2 M NaOH. The weight ratio of copolymer to cellulase was varied over the range of 0.25-4(w/w) to change the degree of modification (DM) of modified cellulase. The DM was defined as the ratio of modified amino groups of the cellulase to the total amino groups of native cellulase. Unmodified amino groups of the cellulase were determined with trinitrobenzene sulfonic acid (TNBS) [Habeeb, 1966].

3. Stability and Solubility in Organic Solvent

Water soluble solvents such as ethylalcohol and acetone and water insoluble solvents such as benzene and toluene were used in this study. The solutions which contained soluble solvent and modified cellulase and reduced sugar were mixed for 12 hours at 4℃ and centrifuged for 5 minutes at 2000 rpm (1000 g). After centrifugation was lyophilized the supernant liquid was obtained. The residue powder was dissolved in buffer solution for assay of the remaining sugar and the activity of the soluble components. Solubility was defined by the ratio of remaining activity in supernatant to initial FPase activity. In the case of water insoluble solvent, the activity of bottom-phase was measured directly, after mixing solution with 20% organic solvent and centrifugation. The activity of organic solvent phase was measured as the same way as mentioned in water soluble solvent. Stability against water insoluble solvent was defined as the ratio of the total remaining FPase activity in the two phases to the initial FPase activity. Partition coefficient of organic solvent, which indicated solubility of the modified cellulase in the solvents, was defined as the ratio of the FPase activity of modified cellulase in the organic solvent phase to that of the water phase.

RESULTS AND DISCUSSION

1. Relation between the Activity of Modified Cellulase and DM

Modified cellulase of different DM was prepared by varying

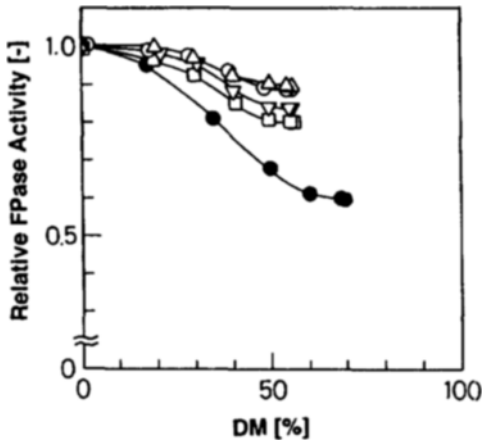


Fig. 2. Relative FPase activities of the cellulase (Onozuka R-10) modified with copolymers against degree of modification (DM).
○ AKM-2010, △ AKM-1511, ▽ AKM-1015, □ AKM-0531, ● Maleic acid anhydride.

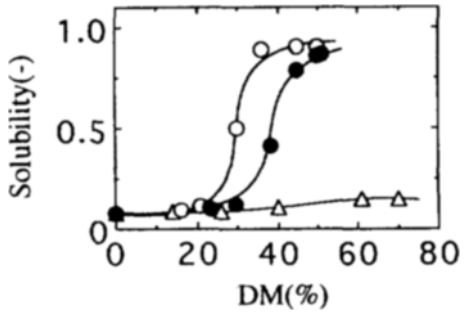


Fig. 3. Solubility of modified cellulase versus degree of modification, control values.
cellulase Acremonium, 4.2 g/L; acetone solution, 80%(v/v). modifier; △ maleic acid anhydride, ○ AKM-1511, ● AKM-0531.

the amount of copolymer added to the enzyme solution, Cellulase Onozuka R-10 was modified with four types of copolymer, which have different degrees of polymerization of EO units in the polyoxyethylene (POE) chain. The relationship between the DM and the FPase activity relative to that of native cellulase is shown in Fig. 2. A DM value of zero corresponds to native cellulase. As the DM increased, the relative FPase activities slightly decreased. In the case of modification with copolymers, maximum modification degree was 55% in all cases, and FPase activities retained more than 80% of the native cellulase activity. The maximum DM by MAA was greater than that of synthetic copolymers, yet the relative activities of modified cellulase with synthetic copolymers were much better than that with MAA at the same DM. The different types of cellulases showed also a similar patterns of relationship between remaining activities of modified cellulase and DM. From these results, it was concluded that synthetic copolymers of higher molecular weight than MAA, were more difficult than MAA to react with amino groups of cellulase due to the steric hindrance of POE chains. However, POE chains of the copolymer, which make a hydrophilic environment, played an important role in the buffering action against denaturalization of enzyme. As a result, POE chains aided the enzyme to maintain high remaining activity.

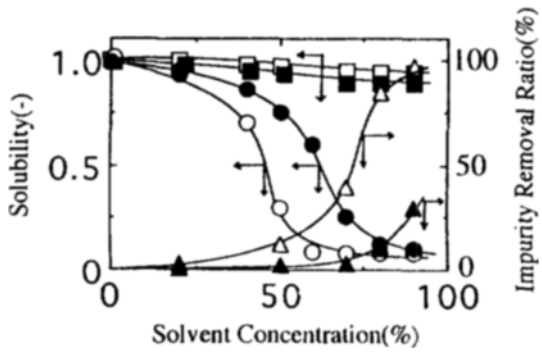


Fig. 4. Solubility and impurity removal ratio against solvent concentration in water-soluble solvents.
Control values: cellulase Onozuka 3S, 3.1 g/L; modifier, AKM-1511; DM, 40%; modified cellulase in; □ acetone, ■ ethylalcohol: native cellulase in; ○ acetone, ● ethylalcohol: impurity removal ratio in; △ acetone, ▲ ethylalcohol.

Table 3. Characteristics of modified cellulase with AKM-1511 in acetone solution

	Modified cellulase		Solubility* [-]	Impurity removal ratio [%] Acetone solution [%(v/v)]		
	DM [%]	Relative initial activity [-]		50%	80%	90%
Onozuka 3S	40.0	0.90	0.94	12.9	85.0	91.2
Y-NC	48.4	0.88	0.89	10.6	65.0	80.0
Acremonium	45.0	0.92	0.90	4.7	68.8	75.5

*: in acetone solution 90 %(v/v)

2. Separation of Modified Cellulase Using Solubility Difference in Water-soluble Organic Solvent

Solubility of modified cellulase in water-soluble organic solvent was studied to separate cellulase from native cellulase and impurities after saccharification. Onozuka 3S which shows the highest reducing sugar concentration was modified with AKM-1511. Modified cellulase was mixed in 80%(v/v) acetone solution, and the relationship between the solubility and DM was investigated as shown in Fig. 3. Native cellulase (DM=0) was almost completely precipitated. In the case of modified cellulase with copolymer, as the DM increased, the solubility rapidly increased, while modified cellulase with pure MAA almost precipitated. The solubility of modified cellulase of 40% DM and impurities removal ratio against solvent concentration in water solvents were showed in Fig. 4. The impurity removal ratio was defined as the ratio of precipitated reducing sugar concentration to the intial value. As the solvent concentration increased, native cellulase precipitates readily, while modified cellulase was always soluble in mixed solvents. In contrast, as the solvent concentration increased, reducing sugar precipitates was removed by more than 90% at above 90%(v/v) acetone concentration. The other modified cellulases, TP-60 and Acremonium, also showed a excellent solubility and high impurity removal ratio. Table 3 showed the characteristics of modified cellulases with AKM-1511 in acetone solution. These results showed that modified cellulase was found to be soluble in acetone and ethylalcohol solution, leaving the impurities in the fermentation. It was also expected that both cellulase and products could be separated after saccharification.

3. Stability and Solubility of Modified Cellulase in Water-

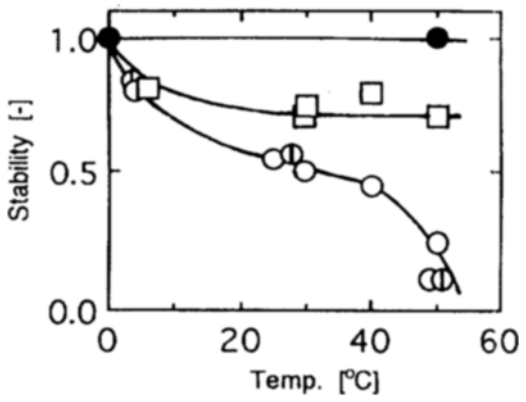


Fig. 5. Stability of native and modified cellulases against organic solvent versus incubation temperature.
Control values: cellulase, 0.2 mg/mL; mixing time, 20 minutes; DM, 44%; solvent, 20%(v/v). Native (○ Benzene, ◑ Toluene, ● Pure water), ACM (□ Benzene).

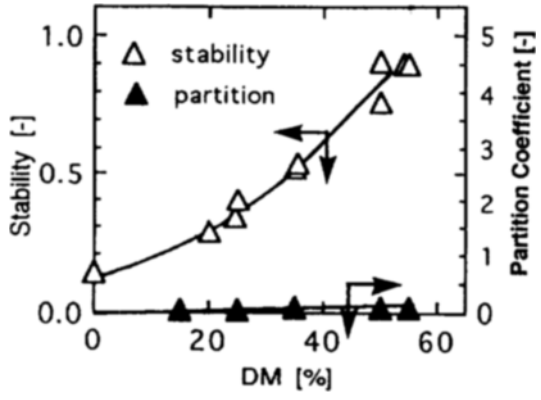


Fig. 6. Stability and partition coefficient of modified cellulase with AKM-1511 versus the DM.
Control values: solvent, benzene 20%(v/v); temperature, 50℃; mixing time; 20 minutes.

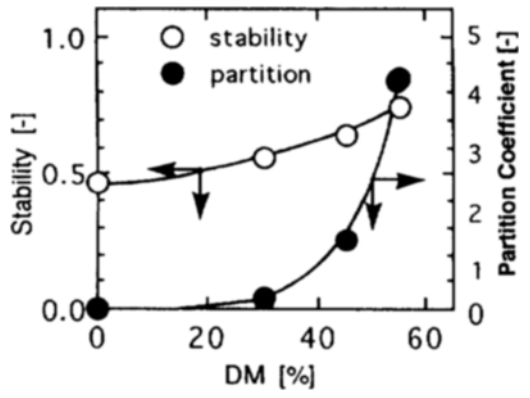


Fig. 7. Stability and partition coefficient of modified cellulase with ACM-1611 versus the DM.
Control values: solvent, benzene 20%(v/v); temperature, 50℃; mixing time; 10 minutes.

Table 4. Stability in water/benzene, toluene

	Native		AKM		ADM		ACM	
min	B	T	B	T	B	T	B	T
30	0.45	0.46	0.98	0.97	-	0.92	0.75	0.82
60	0	0	0.90	0.90	-	0.90	0.68	0.75
120	-	-	-	-	0.78	-	0.62	0.68

DM: 40% Temperature: 40℃
B: benzene 20 vol% T: toluene 20 vol%

insoluble Organic Solvent

Solubility in water-insoluble organic solvent was thought to extract modified cellulase from native cellulase. In this case, the stability is the preliminary condition in using this method. The stability of native and modified cellulase against organic solvent with temperature were shown in Fig. 5. The native and modified cellulases with ACM-1611 were mixed with 20%(v/v) benzene and toluene solution for 20 minutes. Though native cellulase showed excellent stability in pure water, it showed rapid deactivation with the increase of temperature in organic solvents and did not show solubility in the solvent phase. On the contrary, modified cellulases with ACM-1611 showed higher stability than native cellulase and dissolved in the solvent phase. These results suggested that the copolymer of modified cellulase surface played a buffering action against denaturation by attack of the organic solvent.

Stability and partition coefficient of modified cellulase with AKM-1511 which was synthesized with pure EO in POA chain, against the change of DM was showed in Fig. 6. As the DM increased, the stability rapidly increased and showed more than 0.8 at above 50% DM, while the partition coefficient was very low and slowly increased with increase of DM. It was thought that modified cellulase with hydrophilic copolymer was difficult to dissolve in water-insoluble organic solvents. Therefore, the case of hydrophobic modified cellulase was considered. Mixed copolymer containing both EO and PO was very useful for varying the relative hydrophobicity of modified cellulase. The results of modified cellulase with ACM-1611 which had 80% PO concentration in POA chain were shown in Fig. 7. Modified cellulase was stable and the partition coefficient rapidly increased with increase of the DM. Thus, the modified cellulase with hydrophobic copolymer could be soluble in water insoluble organic solvent and it could be possible to separate the modified cellulase and native cellulase using the solubility in organic solvent. The stability of modified cellulase with 50% of DM at 40℃ in water/benzene and water/toluene was shown in Table 4. Native cellulase almost lost its activity after 1 hour, while the remaining activity of modified cellulase with AKM or ADM showed about 90%. In the case of ACM, remaining activity is a little below 70% after 1 hour, but it maintained more than 60% after 2 hours. On the other hand, modified cellulase with hydrophobic copolymer, ACM-1611, showed high solubility in organic solvent phase while the case of ADM showed a similar results of AKM and scarcely moved to organic solvent phase. The solubilities in both benzene and toluene are almost same. From these results, modified cellulase with ACM-1611, which consists of 80% PO concentration in POA chain, improved the stability and solubility in water-insoluble organic solvent, but slightly deactivated. Modified cellulase may be deactivated by the excessive solubilization. Deactivation occurred in the structure conformation of hydrophobic function of cellulase surface. Therefore new amphiphilic copolymer which added hydrophobicity to modified cellulase without structure conformation of cellulase could improve effective solubility in organic solvents.

CONCLUSION

Characteristics of modified cellulase by using amphiphilic copolymer in organic solvents was studied and the following results were obtained.

1. Amino groups of the cellulase were easily coupled with the MAA functional group of the POA-derivative under the condition of pH 8.0 and 4℃. The activity of modified cellulase retained more than 80% of the native cellulase activity at the maximum DM of 55%.

2. Modified cellulase with more than 40% of DM was soluble in water-soluble solvent such as acetone and ethanol, while native cellulase and reducing sugar was precipitated under high solvent concentration.

3. As the DM increased, the stability of modified cellulase against water-insoluble solvent rapidly increased, and the modified cellulase with hydrophobic copolymer showed high partition coefficient in solvent phase.

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