

## REACTIVE AQUEOUS TWO-PHASE PARTITION OF CELLULASE

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**Abstract**—Reactive aqueous two-phase partition of cellulase by modification with amphiphilic copolymer was studied. The modifier of cellulase could be directly used as a polymer of two-phase system. The maleic acid anhydride functional group of the synthetic copolymer covalently coupled with the amino groups of cellulase molecule. The degree of modification of the amino groups was controlled by the types of synthetic copolymer or the weight ratio of a synthetic copolymer to cellulase. As the degree of modification of cellulase increased, the modified cellulase moved to the copolymer phase of the copolymer/dextran two-phase system.

## INTRODUCTION

Enzymatic conversion of waste cellulosic materials to lower molecular chemicals is a very promising process because cellulose is the most abundant renewable resource. However one of the major problems associated with this process is the high cost and consumption of cellulolytic enzymes [2]. From this view point of some important points have to be considered; (1) production of high active cellulase, (2) effective separation and purification of cellulase from culture broth, (3) stability of activity against reaction environment such as temperature, pH and organic solvent, (4) high conversion of substrate during saccharification and, (5) recovery of free cellulase from product for reuse. In our previous work [3], cellulase was modified with synthetic copolymers, such as polyoxalkylene glycol (PAG) derivative, to solve the above-mentioned problems. The PAG derivative copolymer with maleic acid anhydride (MAA) functional group could modify cellulase without so much deactivation. Fig. 1 shows the process of cellulose hydrolysis by cellulase. Modified cellulase with synthetic copolymer plays important roles in improving of this process. The modified cellulase displayed a high stability of activity against temperature and pH, and showed 30% greater conversion of filter paper as substrate at 90 h than the native

cellulase did [3]. In addition, the modified cellulase can be recovered from the hydrolytic residue using solubilization in organic solvents [4]. It may be expected that the cellulase modification with amphiphilic copolymer is useful to partition cellulase into the copolymer phase using the reactive aqueous two-phase system.

In this study the reactive aqueous two-phase partition of cellulase was considered by using the cellulase modification process with the new amphiphilic copolymer of polyoxyalkylene glycol and maleic acid anhydride. The modifier of cellulase could be directly used as a polymer of the two-phase system.

## EXPERIMENT

#### 1. Enzyme source and activity

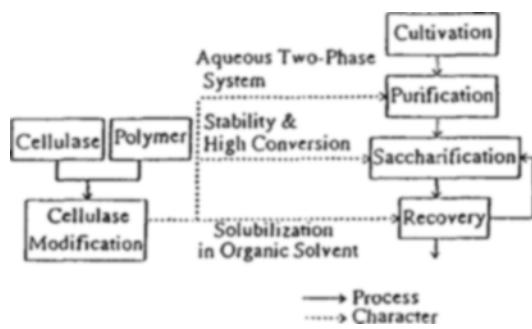


Fig. 1. Conception of modified cellulase in cellulose saccharification process.

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Table 1. Characters of cellulase

Origin	Product name	Protein* (%)		Reducing sugar(%)	Fpase** activity
		Onozuka R-10	Onozuka 3S		
<i>T. viride</i>	TP-60	29.7	8.9	37.5	0.23
	Y-NC	34.1	32.4	60.0	0.44
	Acremonium	21.1	34.6	37.9	0.38
<i>A. niger</i>				26.6	0.19
<i>A. cellulolyticus</i>				0.40	

\*: Specific activity (Unit/mg, protein) was assayed at pH 5.2 and 50°C.

\*\*: Total protein of cellulase was determined by the Lowry method with bovine serum albumin as a standard.

As listed in Table 1, five products of cellulase from three originals, *Trichoderma viride*, *Aspergillus niger* and *Acremonium cellulolyticus*, were used for this experiment. Onozuka R-10, Onozuka 3S and Y-NC were purchased from Yakult Co., Japan. YP-60n and *Acremonium* were kindly provided from MEIJI Ltd., Japan. The protein contents depend on the types of cellulase and the maximum available content is 34.6%. Impurity such as reducing sugar exists also in excess in these products of cellulase. The activity of these cellulases was represented by FPase activity. The FPase activity was assayed as reported by Mandels et al. [5] with FP-5C (Tokyo Roshi Ltd., Japan) as a substrate and represented by a filter paper unit (FPU) which produced 1.0  $\mu$  mole of reducing sugar from the substrate per minute. The reducing sugar was determined by the dinitrosalicylic (DNS) acid method [6].

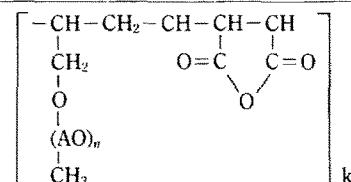
## 2. Synthetic copolymer for cellulase modification

Synthetic copolymers are listed in Table 2. The alternating copolymers (Nippon Oil & Fats Co., Japan) consist of PAG alkylallylether and MAA. The MAA functional group of copolymer covalently coupled with the amino groups of cellulase. These copolymers are characterized by  $k$  which indicates the degree of copolymerization, and by  $n$  which indicates the number of alkylene oxide (AO). The AO consists of ethylene oxide (EO) and propylene oxide (PO). A series of AKM copolymers, which consist of 100% EO in PAG chain, have different values of  $k$ . As  $k$  increases, the MAA number of the copolymer increases. The hydrophobic properties of copolymer were varied by changing the value of EO/AO. As the value of EO/AO decreases, the hydrophobic properties of the copolymer increase and cloudy point (CP) decrease. These hydrophobic copolymers have a similar  $k$  value, from 10 to 11.

### 3. Reactive two-phase partition of cellulase with modification

Table 2. Characteristics of synthetic copolymers

	EO/AO*(wt%)	n	k	MW	CP(°C)**
AKM-0531	100	10	30	18,000	100>
AKM-1015	100	19	14	14,000	100>
AKM-1511	100	33	10	16,000	100>
AKM-2010	100	41	11	21,000	100>
AEM-1511	40	27	10	15,000	40
ADM-1511	30	26	11	18,000	27
ACM-1611	20	29	10	18,000	16



\*:  $(AO)_n$  means the total of EO and PO in PAG chain.  
 AO, alkylene oxide: EO, ethylene oxide: PO, propylene oxide

\*\*: cloudy point (CP) is measured at aqueous solution 10 mg/mL copolymer.

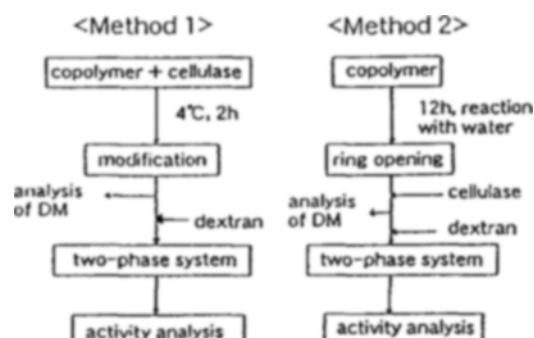


Fig. 2. Experimental methods of reactive two-phase partition

Polyoxyethylene glycol (PEG) and dextran were used as the standard reactive aqueous two-phase system. Four types of PEG (Wako Co., Japan) were used and the average molecular weight (MW) was 1500, 3000, 7500 and 20000, respectively. Also, the average MW of dextran (Wako Co., Japan) was 100000-200000. The reactive aqueous two-phase system was made up of synthetic copolymer and dextran. Two types of method were considered to observe the reaction between copolymer and cellulase as shown in Fig. 2. In method 1, the copolymer and cellulase were reacted at 4°C for 2 h. After reaction of modification, the degree of modification (DM) was determined. Dextran was added to the solution to form a two-phase system and the cellulase activity of the top and bottom phase was

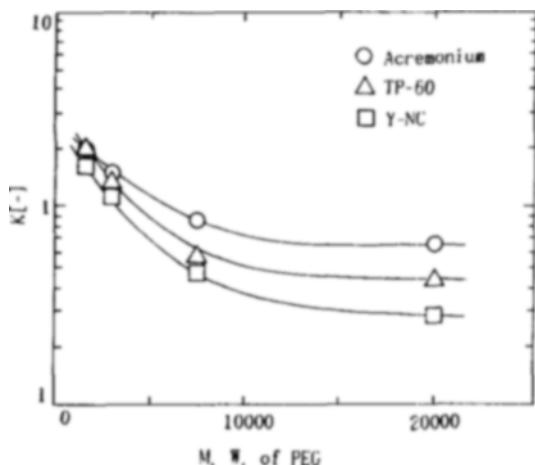


Fig. 3. Relation of partition coefficient to molecular weight of PEG in PEG/dextran system.

Control values: PEG, 8.3%; Dextran, 8.3%; PEG /cellulase, 10 (mg/mg)

analyzed to obtain the value of partition coefficient (K). In method 2, the copolymer was reacted with water for 12 h to open the ring of MAA. Cellulase was then added to the opened ring copolymer solution and DM was determined. The following steps are the same as in method 1. The DM of modified cellulase was defined as the ratio of the unmodified  $-\text{NH}_2$  of modified cellulase to the total  $-\text{NH}_2$  of native cellulase. Amino groups of the cellulase were determined with trinitrobenzene sulfonic (TNBS) acid method [1]. The value of K was defined as the ratio of top phase activity to the bottom phase activity.

## RESULTS AND DISCUSSION

Cellulases were partitioned by the standard two-phase system, namely PEG/dextran system, which is the relationships of K to the average MW of PEG against cellulases from three originals as shown in Fig. 3. The concentrations of PEG and dextran in the two-phase system are equally 8.3%. As the MW of PEG increases, the K values of cellulases always decrease and cellulases move to the dextran phase because of the hydrophilic property of cellulase. However, it is necessary to move cellulase to PEG phase because the enzyme has higher stability in PEG.

Therefore, to solve this problem, a new synthetic copolymer, which has PEG chain and can be expected to react with the cellulase, was considered for a polymer of the two-phase system. To observe the reaction between new synthetic copolymer and cellulase, the

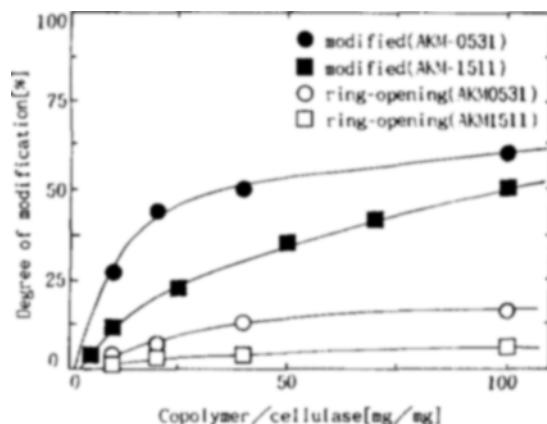


Fig. 4. Degree of modification versus weight ratio of copolymer to cellulase.

Control values: Enzyme, TP-60; Copolymer, 9.6%; Dextran, 9.6%

DM of modified cellulase was analyzed according to the modification condition. The relation of DM to the weight ratio of a synthetic copolymer to cellulase is shown in Fig. 4. Two copolymers, AKM-1511 and AKM-0531, were used. In the case of modification reaction by method 1, as the weight ratio of copolymer to cellulase increases, the degree of modification rapidly increases. Furthermore, the degree of modification of AKM-0531 was higher than that of AKM-1511. On the other hand, the opened ring copolymer by method 2 scarcely modified the cellulase. From these results, it is observed that synthetic copolymer with MAA functional group can modify the cellulase under the condition of method 1 and the DM of modified cellulase can be controlled by varying the weight ratio of the copolymer to cellulase. Therefore, all the experiments concerning cellulase modification were done using method 1. Also, it is expected to control the DM of the cellulase by changing the types of the copolymer. To observe the effect of the types of copolymer, a series of AKM copolymer, which consist of 100% EO in PAG chain, is used. These copolymers are hydrophilic and characterized by the k value. The value of k is the degree of copolymerization and means the number of MAA functional groups in the copolymer molecule. Fig. 5 shows the value of DM and K of cellulase in copolymer/dextran two-phase system against the number of MAA function of copolymers. In this case, the weight ratio of the copolymer to cellulase is constant. As the k value of the copolymer increases, DM increases. Therefore, the DM of the modified cellulase can also be controlled by the number of MAA

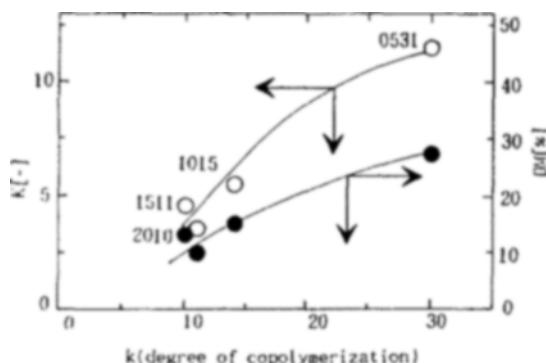


Fig. 5. **K** and **DM** against the number of maleic acid anhydride of copolymer.

Control values: Enzyme, TP-60; Dextran, 9.6%; Copolymer, 9.6% Copolymer/cellulase, 10 (mg/mg);  $k$ : AKM-2010, 11; AKM-1511, 10; AKM-1015, 14; AKM-0351, 30

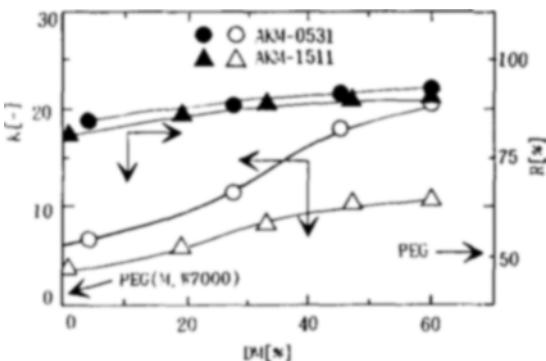


Fig. 6. **K** and **R** against **PO** concentration in **PAG** chain.

Control values: Enzyme, TP-60; Copolymer, 8.3%; Dextran, 8.3%; Copolymer/cellulase, 40 (mg/mg).  $PO/AO$  (wt%): AKM, O; AEM, 60; ADM, 70; ACM, 80

functional groups in a copolymer molecule. In the partition of cellulase, as the  $k$  value of the copolymer increases, the value of  $K$  also increases. Therefore from these results, cellulase can be partitioned into synthetic copolymer phase.

On the other hand, the hydrophobic property of the copolymer can be changed by varying the concentration of PO in a PAG chain. The effects of hydrophobic copolymer on  $K$  were considered. The relationships of  $K$  and recovery rate ( $R$ ) to the hydrophobic property of copolymers are shown in Fig. 6. The value of  $R$  was defined as the ratio of the cellulase of the top phase to the total cellulase. In this case, the weight ratio of the cellulase to the copolymer is 40 for all

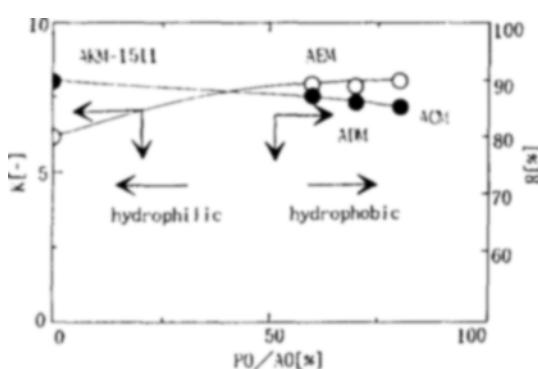


Fig. 7. Relation of **K** and **R** to degree of modification.

Control values: Enzyme, TP-60; Copolymer, 9.6%; Dextran, 9.6%

and the value of  $DM$  is about 30%. As the value of  $PO/AO$  increases, hydrophobic property increases. As the hydrophobic property of the copolymer increases, the  $K$  value slightly increases, while  $R$  of cellulase slightly decreases. The decrease of  $R$  value can be explained by the volume of the copolymer phase. The volume of the copolymer phase decreases incrementally with the increase in hydrophobic property. Therefore, to obtain a high recovery rate of cellulase, hydrophilic copolymer is more useful than hydrophobic copolymer. It has been mentioned that the degree of modification may affect the  $K$  value. Therefore, the cellulase partition against  $DM$  of cellulase was considered. The relationships of  $K$  and  $R$  of the cellulase to the  $DM$  are shown in Fig. 7. The hydrophilic copolymers, AKM-1511 and AKM-0531, were used. In the case of PEG which has a similar molecular weight to the copolymer, the values of  $K$  and  $R$  are 0.54 and 51%, respectively. As the  $DM$  of modified cellulase increases, the value of  $K$  rapidly increases and the  $R$  value of cellulase also increases. From these results, it is found that reactive two-phase partition using the new synthetic copolymers is more useful than PEG/dextran two-phase system. The value of  $DM$  is also an important factor in the reactive two-phase partition. In the same  $DM$  value, the value of  $K$  of AKM-0531, which has a low molecular weight in a unit PEG chain, is higher than that of AKM-1511. Therefore, in the reactive two-phase partition, the copolymer which has sufficient functional groups and PEG chain of low molecular weight is useful to obtain a high  $K$  value.

## CONCLUSIONS

The reactive aqueous two-phase partition of cellulase was studied and the following results were obtained.

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1. The amphiphilic copolymer could be used as a polymer of two-phase system and cellulase is effectively separated by the reactive aqueous two-phase system using the cellulase modification.

2. The degree of modification can be controlled by changing the weight ratio of the copolymer to cellulase or the types of copolymer which have different number of maleic acid anhydride.

3. The partition coefficient is strongly affected by the degree of modification (DM). As the DM increases, the modified cellulase moves to the copolymer phase and recovery rate of cellulase increases.

## NOMENCLATURE

EO/AO : ethylene oxide concentration in PAG chain  
[%]

DM : degree of modification [%]

k : the number of copolymerization

K : the value of partition coefficient

R : recovery rate [%]

(AO) : the total of EO and PO in PAG chain

CP : cloudy point [°C]

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