immersion in chloroform following aq. NaCl treatment. The remarkably reduced C-H stretching peaks (2917 cm^{-1} and 2850 cm^{-1}) show the removal of residual template amines. Meanwhile, it is noticed that the peaks of carbonyl region remain intacted. From the relative density of C, N, and O determined from XPS measurement as described above, the extent of the amine removal was estimated to be completed.

Conclusion

A LB method for two-dimensionally crosslinked polymer network employing an tert-amine as template was applied to the system of an itaconic acid copolymer and poly(allylamine). The formations of a three-component polyion-complexed monolayer, a covalent crosslinking in the LB film, and subsequent removal of template amine, which are the basic steps for the method, could be established in an itaconic acid copolymer and PAA system. The template LB method is supposed to be applicable to other polymer systems.

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Adsorption Characteristics of Endo II and Exo II Purified from *Trichoderma viride* on Microcrystalline Celluloses with Different Surface Area

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The adsorption behaviors of two major components purified, endo II and exo II, from *Trichoderma viride* were investigated using microcrystalline cellulose with different specific surface area as substrates. Adsorption was found to apparently obey the Langmuir isotherm and the thermodynamic parameters, ΔH, ΔS, and ΔG, were calculated from adsorption equilibrium constant, K. The adsorption process was found to be endothermic and an adsorption entropy-controlled reaction. The amount of adsorption of cellulase components increased with specific surface area and decreased with temperature and varied with a change in composition of the cellulase components. The maximum synergistic degradation occurred at the specific weight ratio of the cellulase components at which the maximum affinity of cellulase components obtains. The adsorption entropy and enthalpy for respective enzyme system increased with specific surface area increase. The adsorption entropy was shown to have a larger value with enzyme mixture.

Introduction

One of the most difficult and undeveloped area in enzyme kinetics relates to enzymatic reactions involved with insoluble substrates. Since native cellulosic materials are water-insoluble solid substrates, the cellulose-cellulase system is heterogenous, and the hydrolysis reaction involves several steps. Among these, the adsorption of cellulase is very important because adsorption is a prerequistic step in enzymatic hydrolysis reaction of cellulose and detailed studies on adsorption may lead to a better understanding of mechanism of the enzymatic hydrolysis process.[1-3]

Recently, some important observation on cellulase adsorption have been reported.[4-9] These include the effects of various pretreatments of cellulose and sample preparation on the adsorption of cellulase components, the relation between the specific surface area of the cellulose particle and the adsorbed amount of soluble protein, and the competitive adsorption of cellulase components. Nevertheless, the adsorption characteristics of a mixture of various cellulase components and the mechanism of cellulase adsorption on cellulosic material are still not fully understood. A good understanding of adsorption phenomena concerning cellulase components may provide some clues to the true reaction mecha-

nism and the synergism of the cellulase complex.

Previously, we have described the adsorption behaviors of endo- and exo-type cellulase, partially purified from fungus *Trichoderma viride* on microcrystalline cellulose. It was shown that the maximal synergistic degradation occurs at the specific weight ratio of cellulase components at which the maximal affinity of cellulase components obtains.[10]

In this work, the relationship between the adsorption modes of three cellulases (endo II, exo II, and their mixtures) was investigated using microcrystalline cellulosic substrate with different surface area. In addition, the relationship between the thermodynamic parameters of cellulase adsorption and the surface area of the substrates is investigated.

Experimental

Enzyme. Cellulase powder of *Trichoderma viride* was a gift from Meiji Seika Kaisha Ltd., Japan. Two major cellulase components, such as endo II and exo II, were isolated from a commercial cellulase derived from the fungus *Trichoderma viride* by a series of chromatography procedures involving Bio-Gel P 10, Bio-Gel P 100, DEAE-Sephadex C 50, and Avicel pH 101, as reported previously[11]. The average molecular weights determined by SDS-polyacrylamide gel electrophoretic analysis were 47,000, 58,000 for endo II and exo II, respectively.[11].

Determination of Crystallinity Index. Cellulose powder was pressed into the specimen holder of Rygaku Denki gonimeter diffractometer. Diffractometer traces were obtained using nickel-filtered copper radiation for a range of 2θ from 10 to 30. The crystallinity index (CrI) was defined by Segal *et al.*[12] as follows:

$$\text{CrI}(\%)=[I_{002}-I_{am})/I_{002}]\times 100 \quad (1)$$

where I_{002} is the intensity of the 002 at 22.6 (θ) and I_{am} is the intensity at 18.5 (θ). I_{002} peak is proportional to the sum of both crystalline and amorphous fraction and I_{am} is proportional to the amorphous fraction (Figure 1).

Hydrolysis of Cellulose by Cellulase Components. Enzymatic hydrolysis of cellulose was carried out in 50 mL polyethylene vials in thermostated water bath at 50 ℃, subjected to reciprocal shaking at 120 strokes/min. The pH was adjusted to 5.0 with 0.01 M sodium acetate buffer. The concentration of substrate was 5% in a total volume of 10 mL. The activity of the cellulase preparation used in this hydrolysis was 0.35 FPU/mL. The amount of reducing sugar, consisting mainly of glucose and cellobiose in the sugar solution produced after enzymatic hydrolysis, was estimated by DNS method.[13] Reducing sugar released was expressed as the corresponding amount of glucose. The extent of a saccharification of hydrolysis was calculated from the formula:

$$\%\ \text{Saccharification}=\frac{S_0-S_t}{S_0}\times 100 \quad (2)$$

The S_0 is initial cellulose concentration and the residual cellulose concentration, S_t, can be calculated from the reducing sugar that was measured in terms of glucose as $S_t=S_0-0.9$ P, where P is the reducing sugar concentration in the hydrolysate. The parameter 0.9 represent the molecular weight ratio of glucan monomer to glucose, *i.e.*, correction factor for water taken up by product (glucose) during hydrolysis.

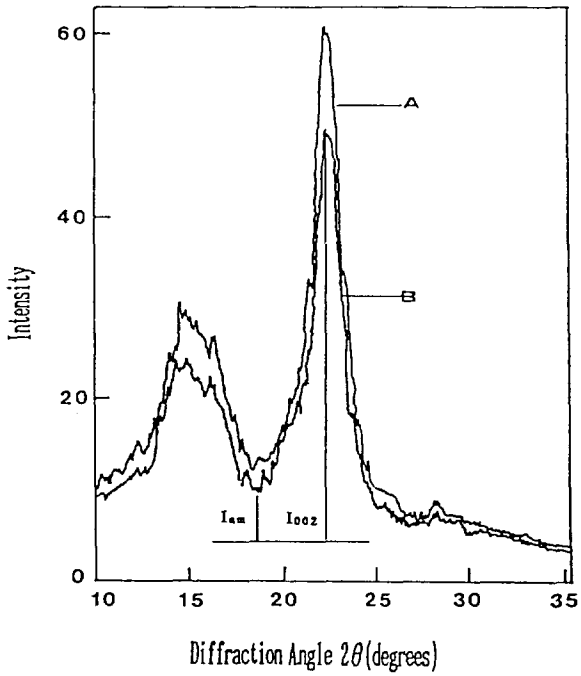

Figure 1. X-ray diffractogram of microcrystalline cellulose. A; Sigmacell 20 (φ20 μm), B; Sigmacell 50 (φ50 μm).

Adsorption of Cellulase on Cellulose. Sigmacell 20 and 50 were used as the cellulose adsorbent. A 50 mg cellulose sample was suspended in 1.0 mL of 0.01 M sodium acetate buffer, pH 5.0, and preincubated at a given temperature of 5-35 ℃ for 30 min. After preincubation, 4.0 mL of a 0.1-2.0 mg/mL cellulase preparation were added. The cellulases employed were endo II, exo II, and mixtures of cellulase components such as $r=6:0$, $r=4:2$, $r=3:3$, $r=2:4$, and $r=0:6$, where r is the weight ratio of endo II to exo II. The reaction mixture was subjected to reciprocal shaking at 120 strokes/min, which is sufficient to attain the adsorption equilibrium, then centrifuged for 20 min at 5,000 rpm. The amount of enzyme in the supernatant was determined by the Lowry method using bovine serum albumin as the reference protein.

Results and Discussion

Effect of Surface area on the Enzymatic Hydrolysis of Microcrystalline Cellulose. The enzymatic hydrolysis is a heterogeneous reaction and is therefore influenced by the structural features of the substrate such as crystallinity, lignin content, and surface area.[14] According to the work of Cowling,[15] and Stone *et al.*,[16] the most important structural feature that influences the enzymatic hydrolysis is the accessibility to cellulose molecules. The effect of surface area of microcrystalline cellulose on the hydrolysis rate is shown in Figure 2. The samples were Sigmacell 20 and Sigmacell 50. These two microcrystalline celluloses had virtually the same crystallinity index (CrI=84.5). Therefore, any differences in hydrolysis rate could be attributed to differences in their specific surface areas. A higher surface area sample resulted in a higher hydrolysis rate. Puri[17] reported that the

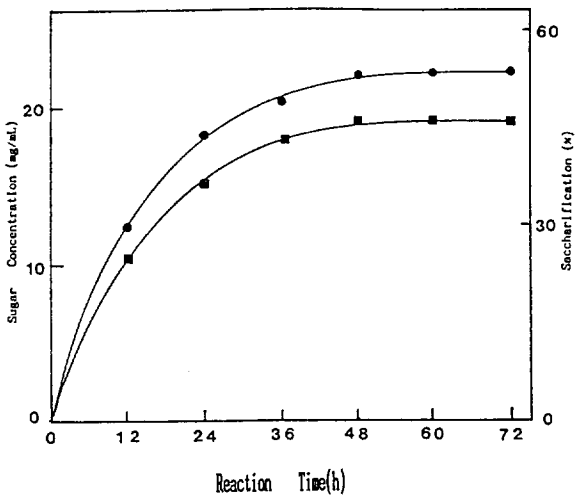

Figure 2. Effect of surface area on the enzymatic hydrolysis. (●) Microcrystalline cellulose (φ20 μm); (■) Microcrystalline cellulose (φ50 μm).

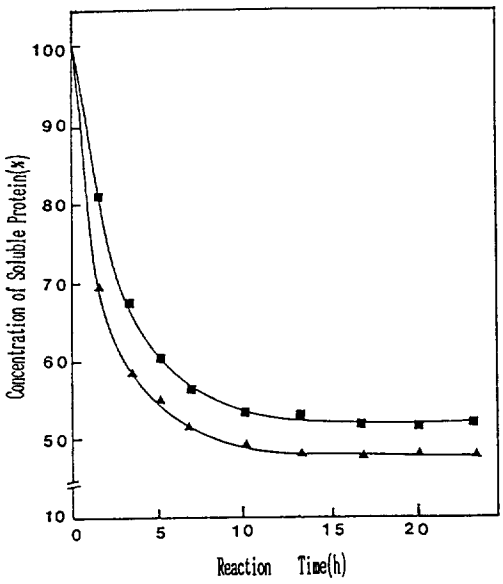

Figure 3. Effect of time on the adsorption of cellulase components on Sigmacell 20 at 5 ℃. (▲) endo II; (■) exo II.

extent and/or rate of hydrolysis is significantly influenced by the surface area of the substrates and the degree of polymerization of the cellulose in combination with other structural features.

Adsorption Behaviors and Kinetics of Cellulase on Microcrystalline Cellulose. Enzymatic reactions involving insoluble substrates consist of several steps: diffusion to the solid surface, adsorption, and finally catalysis. Among these, cellulase adsorption on the susceptible sites of the accessible cellulose surface is a prerequisite step for subsequent catalysis. The binding of enzyme on a specific binding site of substrate may provide the desired specific forces for adsorption of cellulase on the cellulose. It was observed that other proteins such as bovine serium albumin and β-glucosidase were adsorbed onto the cellulose only in negligible

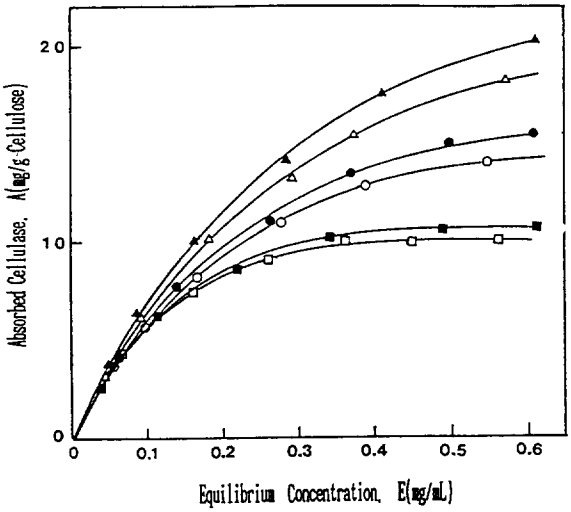

Figure 4. Adsorption isotherms of cellulase components on celluloses at 5 ℃. (1) Sigmacell 20: (●)endo II; (■) exo II; (▲) endo II and exo II mixture. (2) Sigmacell 50 : (○) endo II; (□) exo II; (△) endo II and exo II mixture.

amount.[18] Therefore, the adsorption of cellulase on cellulose seems to be specific. Figure 3 shows the rates of adsorption of endo II and exo II on microcrystalline cellulose . Since the initial adsorption rate is high, a contact time of 15 min was found to be sufficient by which maximum adsorption could take place. Hence, for adsorption studies, the amount of adsorption after a 20 min contact period was taken as the maximum adsorbed value. The adsorption of endo II and exo II on microcrystalline cellulase appears to be completed within 15 min. A plateau during which the amount of cellulase adsorbed per unit area does not change appreciably with further increasing contact time, means that the surface of cellulose is regular and adsorption of cellulase is specific. For quantitative analysis of the effect of the composition of exo II and endo II, the specific surface area of microcrystalline cellulose on adsorption and the adsorption parametes were determined using the Langmuir isotherm Equation. The adsorption of cellulase can be described as follows:

$$[A]=\frac{[A_{max}]K[E]}{1+K[E]} \tag{3}$$

where A_{max} and K are the maximum amount of enzyme adsorbed per unit weight of cellulose and the adsorption equilibrium constant, respectively, and $[E]$ is the concentration of enzyme in liquid phase at adsorption equilibrium. The fraction of adsorption site cannot be measured directly. Thus a concentration of enzyme adsorbed per gram of microcrystalline cellulose was measured. The curves in Figure 4 show that the extent of adsorption is related to the concentration of cellulase at 5 ℃. The assumptions underlying the derivation of the Langmuir isotherm are the independence and equivalence of adsorption sites and adsorbate molecules. However, the cellulase from *T. viride* is multi-component enzyme complex containing three types of enzyme whose synergistic action brings about the degradation of cellulose to glucose. Cellulose may not have uniform adsorption sites,

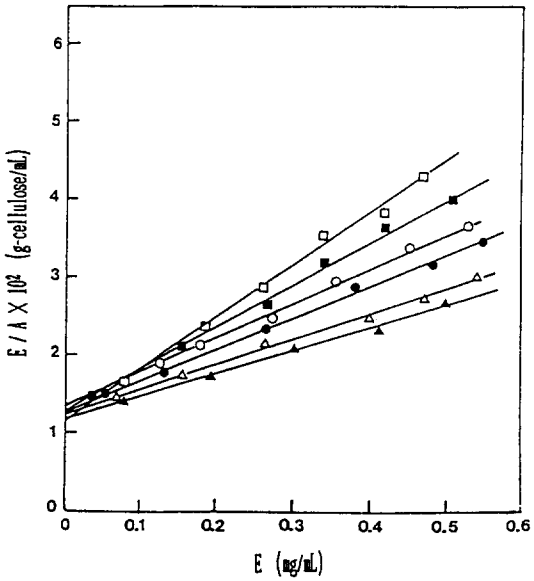

Figure 5. Langmuir plot for the adsorption isotherm of endo II, exo II and mixture of cellulase components at 5 ℃. (1) Sigmacell 20: (●) endo II; (■) exo II; (▲) endo II and exo II mixture. (2) Sigmacell 50: (○) endo II; (□) exo II; (△) endo II and exo II mixture.

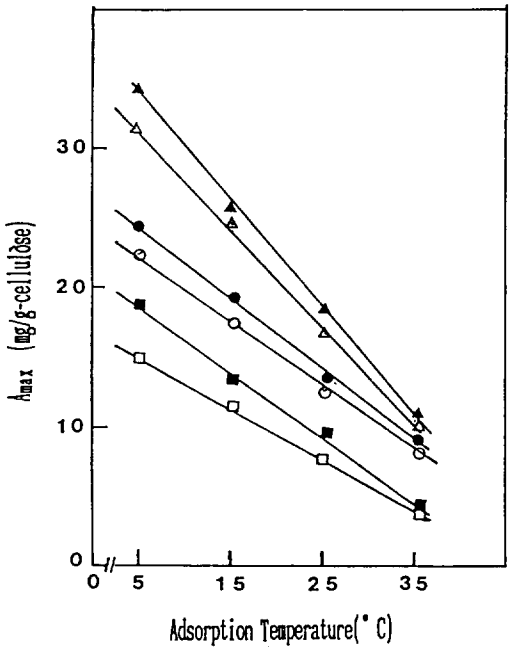

Figure 6. Adsorption temperature dependence of maximum adsorption amount, A_{max}. (1) Sigmacell 20: (●) endo II; (■) exo II; (▲) endo II and exo II mixture. (2) Sigmacell 50: (○) endo II; (□) exo II; (△) endo II and exo II mixture.

although the homogeneity of the particle size of cellulose is very good. Nevertheless, it was found that the adsorption pattern was similar to the Langmuir adsorption pattern. The adsorption isotherm, Eq. (3), can be rearranged as following:

$$\frac{[E]}{[A]} = \frac{1}{K[A]_{max}} + \frac{1}{[A]_{max}}[E] \quad (4)$$

Adsorption equilibrium constant, K and maximum adsorption amount, A_{max} were determined from the plot of $[E]/[A]$, respectively. Then the plot of $[E]/[A]$ vs. $[E]$ gave fairly good straight lines (Figure 5). These results imply that the adsorption of cellulase on microcrystalline cellulose fits the Langmuir isotherm, and are in good agreement with other investigations.[9,10,19~21] Relationship between A_{max} and specific surface area at 5-35 ℃ is illustrated in Figure 6. The values of A_{max} were found to strongly depend on the temperature and the specific surface area. Since Sigmacell 20 and 50 have virtually the same crystallinity index, the differences in A_{max} could be due to differences in their specific surface area. On the other hand, A_{max} decreases rapidly as the temperature increases in all cases. Two factors may be enumerated for why the amount of adsorption decreases with increasing temperature (Figure 6). One is that adsorption of cellulase depends on the temperature, and the other factor is the endo II and exo II desorb into the solution after the hydrolysis reaction is completed at their first adsorption sites. Since the rate of hydrolysis of cellulose increases rapidly with increasing temperature, the rapid hydrolysis rate resulting in a rapid reduction in the number of sites to which endo II and exo II cellulases can adsorb, resulting in a significant rate of desorption. With respect to the interaction between cellulase components, Ryu *et al.*[19] reported that the composition of cellulase components affected

Table 1. Thermodynamic parameters in adsorption of cellulase at 25 ℃

	Endo II		Exo II		Endo II-Exo II mixture	
	20 μm	50 μm	20 μm	50 μm	20 μm	50 μm
ΔG ($JK^{-1}mol^{-1}$)	−30.31	−30.26	−31.46	−31.79	−31.90	−31.69
ΔH ($JK^{-1}mol^{-1}$)	13.22	10.06	11.55	4.51	23.13	19.80
ΔS ($JK^{-1}mol^{-1}$)	146.07	135.30	144.33	121.81	181.31	169.43

the adsorption patterns of cellulase because of the different affinities of the cellulase components. They concluded that the cellulase components adsorbed in a competitive manner. Their results partially agree with the present results that two cellulase components affect the adsorption or desorption of each other. However, the interaction in the experimental results yielded an increase or a decrease of total amount of adsorption (Tables 2 and 3). That is, the amount of adsorption at $r=0.2$ and $r=0.5$ was less than theoretical A_{max}, and at $r=1$, $r=2$, and $r=5$ was more than theoretical A_{max}, where the theoretical A_{max} means the sum of the adsorption amounts of cellulase components which were obtained from the adsorption data of each component. This difference indicates that adsorption action could not be completely explained by competitive adsorption. In case of positive ΔA_{max}, total cellulase affinity toward cellulose may be increased by the interaction between cellulase components, thus increasing the extent of adsorption. However, negative ΔA_{max} values could be explained in terms of the competitive adsor-

Table 2. Differences in maximum adsorption amount, A_{max}, with respect to the composition of cellulase components on Sigmacell 20 at 25 ℃

Enzyme parameter	Endo II	Exo II	Endo II-Exo II mixture													
			$r=0.2^a$			$r=0.5^a$			$r=1^a$			$r=2^a$			$r=5^a$		
T (℃)	$A_{max}{}^b$	A_{max}	Theo. A_{max}	Exp. A_{max}	$\Delta A_{max}{}^c$	Theo. A_{max}	Exp. A_{max}	ΔA_{max}	Theo. A_{max}	Exp. A_{max}	ΔA_{max}	Theo. A_{max}	Exp. A_{max}	ΔA_{max}	Theo. A_{max}	Exp. A_{max}	ΔA_{max}
5	24.44	18.50	19.49	15.78	−3.71	20.48	19.36	−1.12	21.47	34.37	12.90	22.46	27.77	5.31	23.45	25.89	2.44
15	19.21	13.54	14.49	11.02	−3.47	15.43	14.39	−1.04	16.38	25.51	9.13	17.32	21.43	4.11	18.27	21.28	3.01
25	13.52	9.22	9.94	7.84	−2.10	10.65	9.70	−0.95	11.37	18.51	7.14	12.09	16.51	4.42	12.80	15.78	2.98
35	9.25	4.26	5.09	3.04	−2.05	5.92	5.71	−0.21	6.76	10.94	4.18	7.59	10.76	3.17	8.42	10.08	1.66

a: r is weight ratio of endo-II to exo-II. *b*: Maximum adsorption amount of cellulase (mg/g cellulose). *c*: Difference between theoretical (Theo.) A_{max} and experimental (Exp.) A_{max}.

Table 3. Differences in maximum adsorption amount, A_{max}, with respect to the composition of cellulase components on Sigmacell 50 at 25 ℃

Enzyme parameter	Endo II	Exo II	Endo II-Exo II mixture													
			$r=0.2^a$			$r=0.5^a$			$r=1^a$			$r=2^a$			$r=5^a$		
T (℃)	$A_{max}{}^b$	A_{max}	Theo. A_{max}	Exp. A_{max}	$\Delta A_{max}{}^c$	Theo. A_{max}	Exp. A_{max}	ΔA_{max}	Theo. A_{max}	Exp. A_{max}	ΔA_{max}	Theo. A_{max}	Exp. A_{max}	ΔA_{max}	Theo. A_{max}	Exp. A_{max}	ΔA_{max}
5	23.38	15.00	16.40	12.52	−3.88	17.79	15.81	−1.98	19.19	31.17	11.98	20.59	25.30	4.71	21.98	24.39	2.41
15	17.55	11.15	12.22	8.32	−3.90	13.28	12.06	−1.22	14.35	24.45	10.10	15.42	19.43	4.01	16.48	18.36	1.88
25	12.56	7.44	8.29	5.79	−2.50	9.15	8.05	−1.10	10.00	16.55	6.55	10.85	14.76	3.91	11.71	13.31	1.60
35	8.61	4.00	4.77	2.66	−2.11	5.54	4.14	−0.40	6.31	10.27	3.96	7.09	9.75	2.68	7.84	9.85	2.01

a: r is weight ratio of endo-II and exo-II. *b*: Maximum adsorption amount of cellulase (mg/g cellulose). *c*: Difference between theoretical (Theo.) A_{max} and experimental (Exp.) A_{max}.

ption of exo II and endo II cellulase bringing about desorption of endo II by excess of exo II. The maximum value of A_{max} was at $r=1$ and A_{max} decreased with an increase or decrease of r from 1. These results indicate that at a specific weight ratio, the maximum affinity of composition of endo II and exo II exists, and the interaction would be maximized at this condition. Also, Table 1 shows that interaction between endo II and exo II, at specific weight ratio, which may increase in effective adsorption sites, would increase the extent of adsorption. Different values of K were obtained at different temperatures (Figure 7). K is an equilibrium constant for the distribution of enzyme between the cellulose and liquid phase. This indicates that its temperature dependence can be used to determine the value of enthalpy of adsorption, ΔH through the thermodynamic Equation as follows:

$$-\frac{\Delta H}{R}=\frac{d\ln K}{d(1/T)} \tag{5}$$

Eq. (5) shows that a plot of $\ln K$ vs. $1/T$ has a slope equal to $\Delta H/T$. The value of K can be used to obtain the value of free energy of adsorption, ΔG:

$$\Delta G=-RT\ln K \tag{6}$$

The value of ΔG is used with the value of ΔH to obtain the value of entropy of adsorption, ΔS:

$$\Delta S=\frac{\Delta H-\Delta G}{T} \tag{7}$$

Table 1 show the values of enthalpy, free energy, and entropy accompanying the adsorptions which were obtained from Eqs. (5), (6), and (7). The mean molecular weights of endo II and exo II cellulases required to obtain the thermodynamic parameters were 47,000 and 58,000,[11] respectively. It was found that the adsorption process of endo II, exo II, and the composition of endo II and exo II cellulase ($r=1$) was endothermic and adsorption entropy-controlled (Table 1). The heats of adsorption of endo II and exo II are in the range of 4.51-13.22 $JK^{-1}mol^{-1}$, much smaller than that of endo II in combination exo II (19.80-23.13 $JK^{-1}mol^{-1}$). These results indicate that the synergistic action of endo II in combination exo II would affect on the heats of adsorption through the interaction between enzyme components during the adsorption process. Ryu *et al.*[19] indicated that the composition of enzyme components affects the adsorption patterns of cellulase because of the different affinites of the cellulase components. The large negative values of ΔG indicate that the adsorption of endo II, exo II, and the composition of cellulase components ($r=1$) took place spontaneously. Because of the simillar the adsorption free energy of endo II and exo II, there will be a competition between individual enzymes to occupy the available adsorption sites. The values of ΔS are expected to be negative because there is reduction in the translational freedom when cellulase is adsorbed to cellulose. The entropy change, ΔS at $r=1$ was larger than that for endo II or exo II cellulase alone. This result indicates that the composition of cellulase components would affect

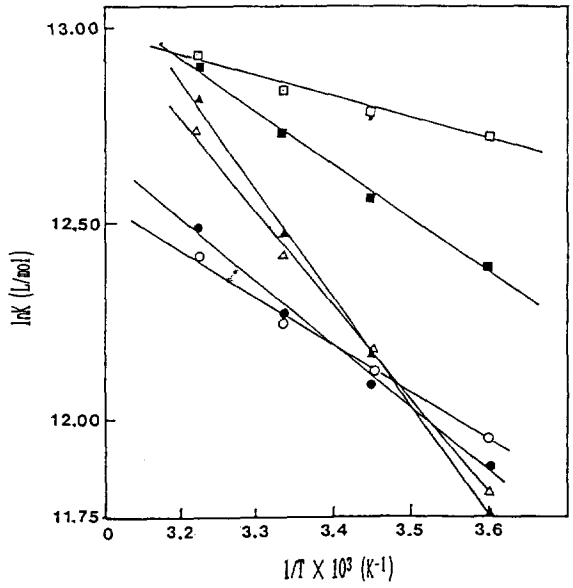

Figure 7. Temperature dependence of adsorption equilibrium constant, *K*. (1) Sigmacell 20: (●) endo II; (■) exo II; (▲) endo II and exo II mixture. (2) Sigmacell 50: (○) endo II; (□) exo II; (△) endo II and exo II mixture.

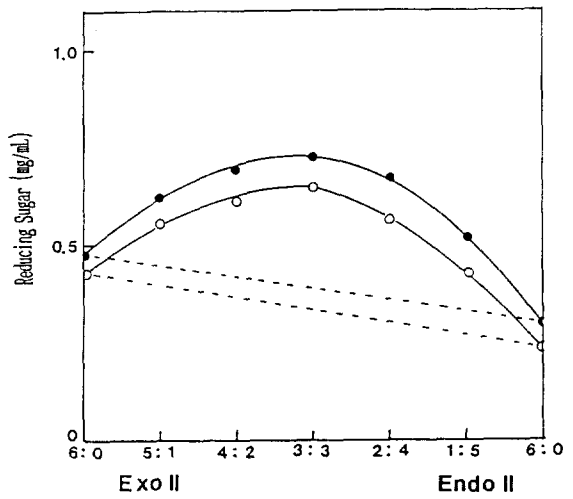

Figure 8. Synergistic degradation of microcrystalline cellulose by various endo II and exo II mixtures. The dotted lines represent the theoretical values expected for non-cooperative degradation. (●) Sigmacell 20; (○) Sigmacell 50.

an entropy change through the adsorption process. It was observed that the entropy of adsorption had a higher positive value with an increase in specific surface area, which may be ascribed to the difference in adsorption sites. Sigmacell 20 and 50 have the same crystalline- to-amorphous ratio but, due to the relatively more effective adsorption sites of Sigmacell 20, the adsorption entropy had a larger value to Sigmacell 20 than to Sigmacell 50. Adsorption entropy of cellulase was increased with specific surface area. The cellulase can adsorb more readily to Sigmacell 20 than 50. This result means that hydrolysis of cellulose depends mainly on the adsorption entropy.

To investigate the relation between the specific weight ratio and synergistic degradation, endo II and exo II were mixed together in several proportions (6 : 0, 5 : 1, 4 : 2, 3 : 3, 2 : 4, 1 : 5, and 0 : 6) and assayed using microcrystalline cellulose as a substrate (Figure 8). Endo II and exo II acted synergistically with microcrystalline cellulose. The synergistic action was dependent on the enzyme composition. A 1 : 1 mixture of the two enzymes was found to be most effective for digestion. It implies that the maximum synergistic degradation occurred at the specific weight ratio of cellulase components at which the maximum adsorption obtained. This result is consistent with the result of Henrissat *et al.*,[20] who reported that a 1 : 1 proportion of CMCase and Avicelase produced maximum synergistic degradation. As a result, it is very likely that the specific adsorption condition need to be present in order to obtain a maximal synergistic degradation.

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