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Effect of Adsorption of Endoglucanase on the Degradation of Microcrystalline Cellulose

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The influence of adsorption of endoglucanases on enzymatic hydrolysis of microcrystalline cellulose was investigated. All the data of adsorption on microcrystalline cellulose were found to obey the Langmuir adsorption isotherm. The values of adsorption affinity for endoglucanases increased in regular sequence of EG IV<EG III<EG I<EG II. It was found that the mechanisms of enzymatic hydrolysis of a crystalline and an amorphous cellulose are different. The hydrolysis of amorphous cellulose with endoglucanase depends on its activity. But the hydrolysis of microcrystalline cellulose with endoglucanase is directly correlated with its adsorption affinity. The major factor related to cellulases that control the difference in the reactivity of the crystalline and amorphous cellulose appears to be the adsorption affinity of endoglucanase on cellulose. From the results of hydrolysis with endoglucanases (EG I, II, III, and IV) in combination with cellobiohydrolase (CBH II), the reducing sugar produced during degradation is increased when the enzyme system contains endoglucanase with high affinity to cellulose.

Introduction

Cellulases are multicomponent enzyme systems which are capable of hydrolyzing cellulose to glucose. Among the best characterized cellulases are those derived from *Trichoderma*.[1~5] The cellulases of *Trichoderma viride* degrade cellulose in a cooperative manner and consist of two cellobiohydrolases (CBH I and CBH II) and at least four endoglucanases (EG I, EG II, EG III, and EG IV), the former releasing cellobiose from the nonreducing ends of the cellulose chain and the latter cleaving internal glucosidic bonds in native cellulose.[6~8] Because of the complex physicochemical properties of the cellulose, the diversity of the enzymes, and the lack of the structure information, the precise mechanism action of those enzymes is not understood.

The cellulases from *T. viride* comprise two or more domains which function independently. A common arrangement is a catalytic domain connected to a cellulose-binding domain (CBD) by a linker.[9~11] This implies that the first step in the enzymatic hydrolysis is adsorption of cellulase binding domain on the surface of the water-insoluble cellulose fibrils. After binding of the enzyme molecules the actual catalytic action, *i.e.*, the hydrolysis of the susceptible glucosidic bonds, takes place. Therefore, the degradation actions of cellulase components can be observed from the viewpoint of adsorption behaviors of cellulase components.

Removal of CBDs reduces the hydrolytic activity of enzymes on microcrystalline cellulose, whereas their activities on soluble and amorphous celluloses are either unaffected or increased. Binding of the CBDs to cellulose is not affected significantly by removal of their catalytic domains.[12~16] It was recently reported that CBD of cellulase disrupted the surface of Ramie cotton fibers and that the catalytic core domain had a smoothing or polishing effect on the surface.[14] Klyosov[17,18] suggested that the defibrillation effect is an inherent property of both CBH and EG, evidenced by their ability to be adsorbed tightly to cellulose. In other words, only those enzymes with high affinity for crystalline cellulose render it susceptible to hydrolysis. Clearly, an understanding of the degradation of cellulose requires an understanding of the roles of the CBDs in the process.

In this study, we investigated the major factor related to cellulases that control the difference in the reactivity of the crystalline and an amorphous cellulose. The influence of adsorption of endoglucanase on the enzymatic degradation of microcrystalline cellulose is also discussed.

Experimental

Enzymes. Major cellulase components, such as endoglucanases (EGs; I, II, III, and IV) and cellobiohydrolase (CBH II), were isolated from a commercial cellulase (Meicelase TP 60, Lot No. CEPB-5291) derived from the fungus *Trichoderma viride* by a series of chromatographic procedures involving Bio-Gel P 10, Bio-Gel P 100, DEAE-Sephadex A-50, SP-Sephadex C 50, and Avicel PH 101.[5] The purified endogluca-

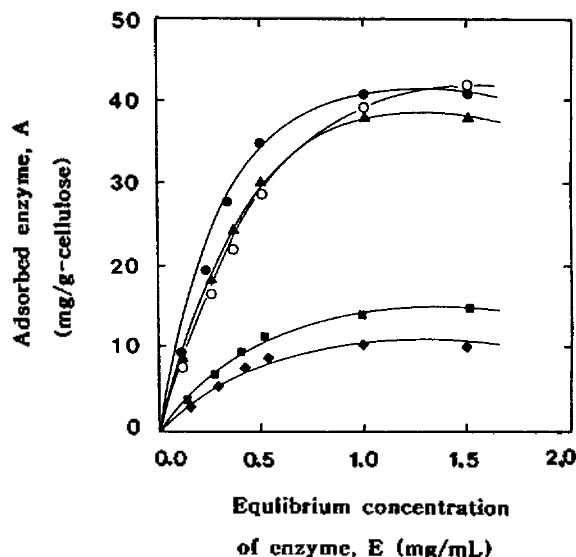

Figure 1. Adsorption isotherms of endoglucanases and cellobiohydrolase II on microcrystalline cellulose at 15 ℃. Symbols : ●, EG I; ▲, EG II; ■, EG III; ◆, EG IV; ○, CBH II.

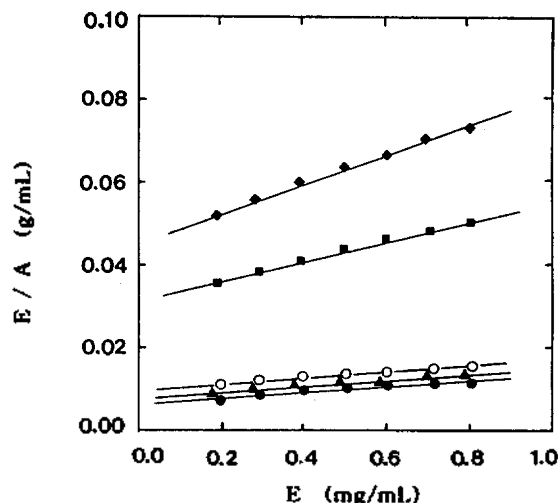

Figure 2. Langmuir plot for the adsorption isotherms of endoglucanases and cellobiohydrolase II on microcrystalline cellulose at 15 ℃. Symbols : ●, EG I; ▲, EG II; ■, EG III; ◆, EG IV; ○, CBH II.

nases (EG I, II, III, and IV) and cellobiohydrolase (CBH II) showed a single band on SDS-polyacrylamide gel electrophoresis. The average molecular weights determined by SDS-polyacrylamide electrophoretic analysis were 52,000, 60,000, 42,000, 38,000, and 62,000 for EG I, II, III, and IV and CBH II, respectively.[5]

Adsorption of enzyme on cellulose. A 50 mg cellulose sample, which was Avicel PH 101 as the cellulose adsorbent, was suspended in 1.0 mL of 0.05 M sodium acetate buffer, pH 4.8, and preincubated at a given temperature of 15 ℃ for 30 min. After preincubation, 4.0 mL of a 0.1-2.5 mg/mL enzyme preparation was added. The enzyme components employed were endoglucanases and cellobiohydrolase. The reaction mixture was subjected to reciprocal shaking at 120 strokes/min for 30 min, which is sufficient time to attain the adsorption equilibrium, and then centrifuged for 5 min at 5,000 rev/min. The amount of enzyme in the supernatant was determined by the Lowry method,[19] using bovine serum albumin as a standard.

Hydrolysis of microcrystalline cellulose. A 100 mg of microcrystalline cellulose (Avicel PH 101) was accurately weighed, immersed in 5 mL enzyme buffer solution at pH 4.8, and incubated at 50 ℃ with shaking at 120 strokes/min. The final enzyme concentration was 0.3 mg/mL. The reaction was stopped after a given time by boiling for 10 min. The amount of reducing sugar released was estimated by the DNS method[20] using glucose as a standard.

Hydrolysis of carboxymethyl cellulose. To 1.0 mL of a substrate (CMC) solution, 0.5 mL of enzyme solution was added and the reaction mixture incubated at 50 ℃ for a given time. The final enzyme concentration was 0.1 mg/mL. The reaction mixture was stopped by boiling for 10 min, and the reducing sugar was determined by the DNS method[20] using glucose as a standard.

Results and Discussion

The adsorption parameters were determined using the Langmuir adsorption Equation in order to obtain a quantitative analysis of the effect of EG I, II, III, IV, and CBH II on adsorption.

The adsorption of enzyme can be described as :

$$[A]=\frac{[A]_{max}\,K_{ad}\,[E]}{1+K_{ad}[E]} \tag{1}$$

where A_{max} and K_{ad} are the maximum amount of enzyme adsorbed per unit weight of cellulose and the adsorption equilibrium constant, respectively; $[E]$ is the concentration of enzyme in the liquid phase at the adsorption equilibrium. Figure 1 shows adsorption as a function of varying enzyme concentration at 15 ℃. Although the adsorption isotherms of EG I, II, III, IV, and CBH II showed characteristic differences, there is a characteristic L shape in all the isotherms. A rapid increase in the amount of enzyme adsorbed onto the cellulose is observed when the concentration of enzyme in solution is increased from 0 to 1.0 mg/mL. At higher concentrations (>1.0 mg/mL), an adsorption plateau is reached.

The adsorption isotherm, Equation (1), can be rearranged as follows:

$$\frac{[E]}{[A]}=\frac{1}{K_{ad}\,[A]_{max}}+\frac{1}{[A]_{max}}[E] \tag{2}$$

K_{ad} and A_{max} were determined from the slope of the plot of $[E]/[A]$ *vs.* $[E]$ using a least-square analysis, respectively. Then the plots of $[E]/[A]$ *vs.* $[E]$ gave fairly good straight lines as shown in Figure 2. These results imply that the adsorption of cellulase enzymes on microcrystalline cellulose fits the Langmuir isotherm and in good agreement with other investigations.[21~27] Of course, it is not surprising that the cellulase with a compact protein molecule follows the Langmuir isotherms as shown in Figure 2. Equation (2) is valid only if the adsorption sites and adsorbate molecules are dependent and equivalent. Nevertheless, adsorption data for

Table 1. Langmuir parameters of cellulase components at 15 ℃

Enzyme	A_{max}[a]	K_{ad}[b] $\times 10^{-4}$
EG I	55.3	19.5
EG II	51.5	20.0
EG III	29.8	4.6
EG IV	61.9	15.5

[a] Maximally adsorbed amount of enzyme components (mg/g cellulose). [b] Adsorption equilibrium constant (L/mol).

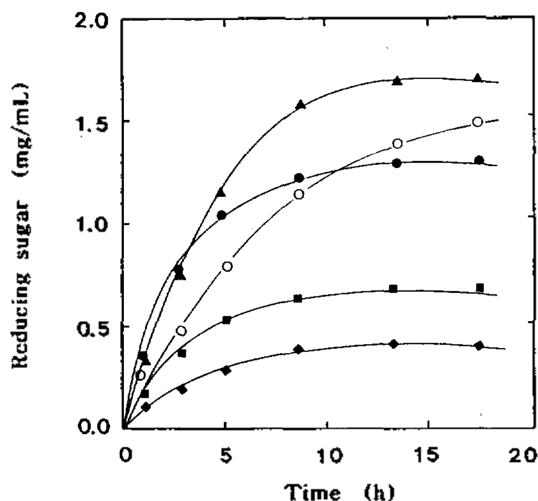

Figure 3. Hydrolysis of microcrystalline cellulose by endoglucanases for 18 hr. Symbols: ●, EG I; ▲, EG II; ■, EG III; ◆, EG IV; ○, CBH II.

cellulase enzymes are known to obey the Langmuir adsorption isotherm, and the adsorption data of the cellulose-cellulase system apparently conformed to Langmuir's reciprocal plot. The adsorption equilibrium constant (K_{ad}) and maximum adsorption amount of cellulase (A_{max}) were shown in Table 1. Here, K_{ad} is an intensive property of adsorption and is a measure for the adsorption affinity; A_{max} is an extensive one, which is proportional to the number of adsorption sites per unit surface and the enzyme accessible specific area of adsorbent. From the K_{ad} values, the endoglucanases and cellobiohydrolase can be divided into two subclasses of enzymes; those enzymes that have high affinity (EGs, I, II, and CBH II) and the others that have moderate affinity (EGs, III and IV) on microcrystalline cellulose.

The time course of sugar concentration for hydrolysis of microcrystalline cellulose is shown in Figure 3. Initial hydrolysis rate was fast, and the reducing sugar was not almost produced after the hydrolysis for 10 hours. The amount of reducing sugar after 18 hours-hydrolysis increased in regular sequence of EG IV<EG III<EG I<EG II. In the case of carboxymethyl cellulose, the rate of hydrolysis is very rapid and large amount of cellulose is quickly consumed within 30 min, and the rate of hydrolysis then decreased as shown in Figure 4. The amount of reducing sugar after 30 min increased in regular sequence of EG II<EG IV<EG II<EG I. The hydrolysis pattern for both microcrystalline and amorphous cellulose with EGs was appreciably different. This result indicates that the mechanisms of enzymatic hydrolysis of a crystalline and an amorphous cellulose are different.

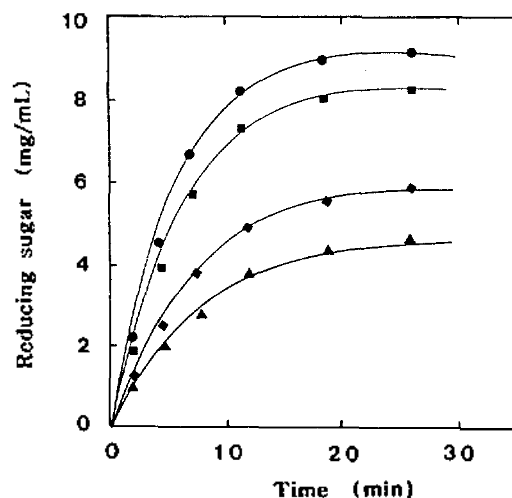

Figure 4. Hydrolysis of carboxymethyl cellulose by endoglucanases at 50 ℃. Symbols: ●, EG I; ▲, EG II; ■, EG III; ◆, EG IV; ○, CBH II.

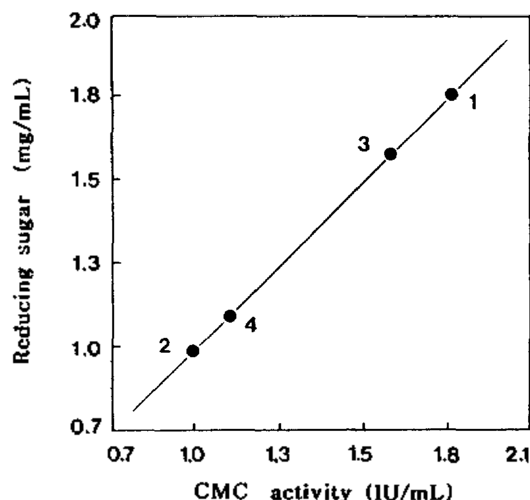

Figure 5. The effect of endoglucanase activity on the hydrolysis of carboxymethyl cellulose at 50 ℃. Symbols: 1, EG I; 2, EG II; 3, EG III; 4, EG IV.

It was expected that the enzyme-substrate ratio would be greatly affected by the physicochemical properties of cellulose such as specific surface area, crystallinity and adsorptivity because the hydrolysis reaction of cellulose was quite specific depending on the physicochemical properties of the cellulose used. To investigate the influence of enzyme activity on the hydrolysis of carboxymethyl cellulose, the sugar production at enzyme concentrations between 1.0 and 1.8 CMC activity (IU/mL) was studied. As shown in Figure 5, the sugar concentration in the hydrolysates after enzymatic hydrolysis for 30 min increased proportionally with an increase in enzyme concentration. This result indicates that the hydrolysis rate of amorphous cellulose catalyzed by cellulase may be well predicted quantitatively, solely from the endoglucanase activity, and that, for efficient hydrolysis of

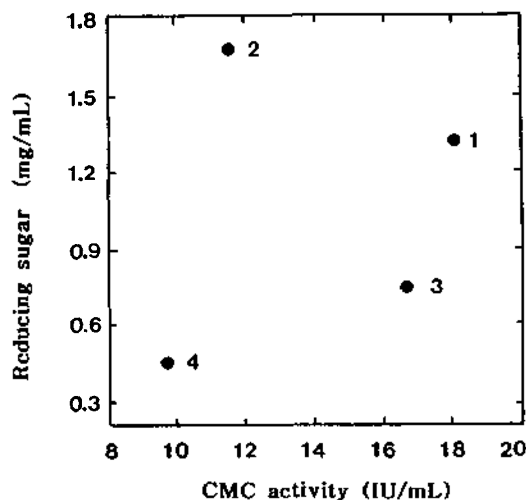

Figure 6. The effect of endoglucanase activity on the hydrolysis of microcrystalline cellulose at 50 ℃. Symbols : 1, EG I; 2, EG II; 3, EG III; 4, EG IV.

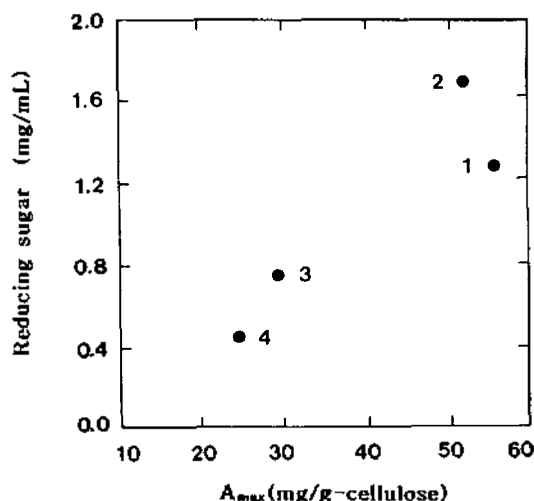

Figure 7. The effect of maximum adsorption equilibrium constant (A_{max}) and reducing sugar produced during the hydrolysis of microcrystalline cellulose at 50 ℃. Symbols : 1, EG I; 2, EG II; 3, EG III; 4, EG IV.

amorphous cellulose, only the quantity (*i.e.*, activity) of a cellulase preparation is important. However, the situation was the reverse with the hydrolysis of microcrystalline cellulose as shown in Figure 6. The hydrolysis of microcrystalline cellulose catalyzed by endoglucanases showed no correlation between the rate of reducing sugar formation and activity of endoglucanases in the reaction system. Therefore, endoglucanase activity is still not a sufficiently pronounced factor to predict the reactivity of crystalline cellulose with respect to cellulose. The cellulase, secreted by the filamentous fungus *Trichoderma viride*, consists of two domains, a globular core domain hydrolyzing the glycosidic linkages of the cellulose, and a wedge-shaped tail domain binding to the cellulose. We have found that, in this respect, the efficiency of endoglucanase adsorption is a necessary factor to predict

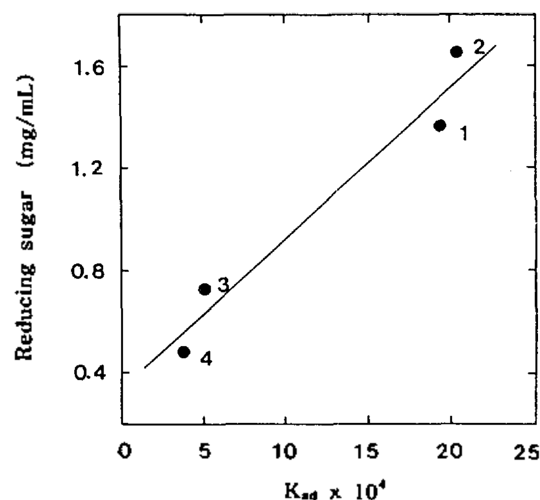

Figure 8. The effects of K_{ad} of endoglucanase on the hydrolysis of microcrystalline cellulose at 50 ℃. Symbols : 1, EG I; 2, EG II; 3, EG III; 4, EG IV.

the reactivity of microcrystalline cellulose. The relation between A_{max} and the amounts of reducing sugar produced is shown in Figure 7. The correlation between those two was limited only a few endoglucanase components. The increase in A_{max} is by no means just by an increase in the reducing sugar. This result indicates the A_{max} is not sufficient to suggest the quality of binding the enzyme to substrate. Figure 8 showed the effect of K_{ad} of endoglucanases on the hydrolysis of microcrystalline cellulose. A fairly distinct correlation was obtained between this parameter and the rate of reducing sugar formation during hydrolysis of the microcrystalline cellulose. So, for all the endoglucanases studied in this work, the hydrolysis of the crystalline cellulose depends on the adsorption equilibrium constant, K_{ad}. The endoglucanases with the higher K_{ad} values produced the more reducing sugar. It was found that the ability of endoglucanase to solubilize crystalline cellulose is directly correlated with its K_{ad}. These results indicate that the affinity of adsorption suggesting the quality of binding the enzyme to substrate plays a crucial role in degrading the microcrystalline cellulose.

The time course of sugar concentration for hydrolysis of microcrystalline cellulose by the EG I, II, III, and IV in combination with CBH II is shown in Figure 9. It was found that the velocity of the hydrolysis of microcrystalline cellulose is proportional to the adsorption ability of the endoglucanase in the enzyme systems. The efficiency of the degradation of microcrystalline cellulose is enhanced when the enzyme system contains endoglucanase with high affinity to cellulose. It seems that tightly bound endoglucanase binds to cellulose at sites where the crystalline structure of the substrate is disturbed and induces a defibrillation of the crystallites as a result of the corresponding mechanochemical effects by the binding domains of the enzyme. The tightly adsorbed endoglucanase can then penetrate intercrystalline regions and open new sites for the action of cellobiohydrolase. These phenomena may be the main reason for the synergism between endoglucanase and cellobiohydrolase. This result is consistent with the results of Klyosov.[17,18] He observed that the stronger the cellulases are adsorbed on crystalline

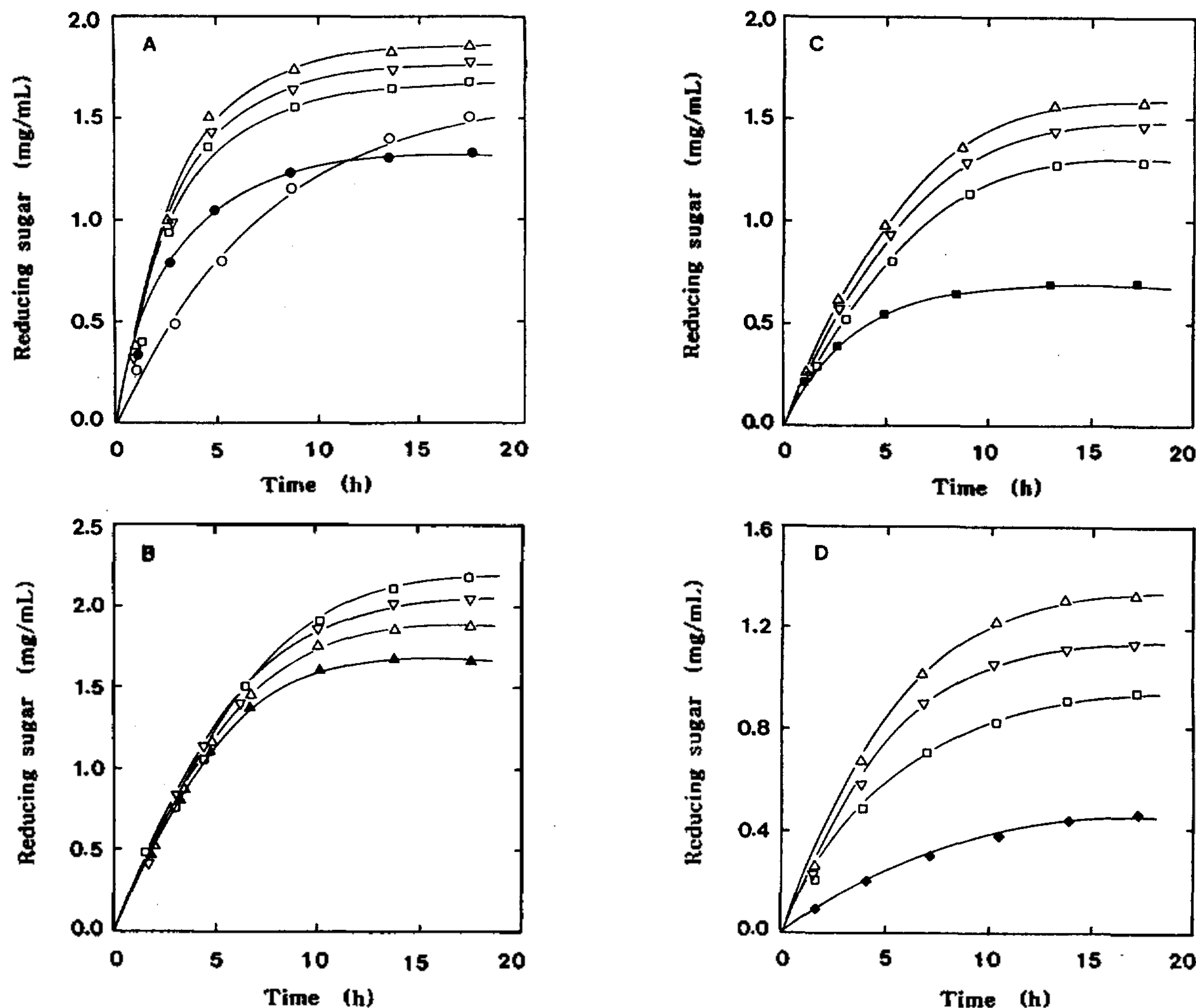

Figure 9. Hydrolysis of microcrystalline cellulose by EG I (A), EG II (B), EG III (C), and EG IV (D) in combination with CBH II for 18 hr. Symbols : ●, ▲, ■, ◆, EGs (EG I, EG II, EG III, EG IV); ○, CBH II; △, r=0.5; !, r=1.0; □, r=2.0. r=weight ratio of endoglucanase to cellobiohydrolase.

cellulose, the higher the rate of reaction and the greater the yield of glucose, irrespective of the duration of the reaction. Moreover, when cellulases are weakly adsorbed, the degree of degradation of cellulose does not exceed 8-9% and corresponds approximately to the share of amorphous cellulose in the substrate. Consequently, it was shown that when the adsorption affinity of the cellulases are strong an almost total hydrolysis of crystalline cellulose takes place.

On the other hand, the velocity of degradation for a specific combination of endoglucanase and exoglucanase, is at least dependent upon two main factors: firstly the ratio in which both enzymes are combined and secondly the adsorption behaviors of these enzymes. The increase of A_{max} and K_{ad} values in the adsorption of EG I, III, and IV in combination with CBH II goes in parallel with its velocity of degradation. The fact that EG I, III, and IV in combination with CBH II gave the maximal degradation, A_{max}, and K_{ad} values at the 2 : 1 ratio of exoglucanase and endoglucanase, may be related to high degree of randomness in cellulose attack of these endoglucanases. The action of the EG I, III, and IV, which jump from chain to chain(multi-chain attack),[5] will not always lead to solubilization, because the chain is fixed in a crystalline matrix. However, it can result in a new chain-end for an exoglucanase to act on, creating new measurable reducing end-groups. Therefore, EG I, III, and IV in combination with CBH need a relatively high cellobiohydrolase concentration to create a "driving force" which is strong enough for the optimal adsorption and degradation. On the other hand, the EG II and CBH II mixture exhibited the maximal degradation and K_{ad} at the 1 : 2 ratio of exoglucanase and endoglucanase. EG II acts according to a single chain multiple attack mechanism[5] and therefore, may provide poor reaction sites for CBH II. Therefore, it is very likely that the high ratio of Endo II in enzyme mixture need to be present in order to obtain a maximal degradation.

We have shown that the mechanisms of enzymatic hydrolysis of a crystalline and an amorphous cellulose are different, and that the major factor related to cellulases that control the difference in the reactivity of the crystalline and amorphous cellulose appears to be the adsorption affinity of endoglucanase on microcrystalline cellulose. These results should help us to predict the reactivity of insoluble substrates in enzymatic degradation.

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Monte Carlo Studies of Argon Adsorbed in 5A Zeolite Cavities

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The grand canonical ensemble Monte Carlo simulations have been carried out to investigate the thermodynamic and energetic properties for the Lennard-Jones system of argon gases adsorbed in 5A zeolites. The adsorption isotherm, the isosteric heat of adsorption and the energy distribution curves are computed at the fixed temperature of 233 K over the bulk pressure range varying from 50 kPa to 400 kPa, and the resulting simulation data are compared with the available experimental values. For temperature and pressure conditions employed in this work the Monte Carlo results are shown to be in reasonable agreement with the corresponding experimental data. Two main peaks in the energy distribution curves are observed due to the energetically distinct regions near the sorption sites of such zeolite cavities.

Introduction

Because of their unique structure with exceptional porosity, zeolite materials have played an important role in industrial applications such areas as purification, separation, catalysis and ion-exchange. In many cases the zeolite structure remains the fundamental factor responsible for these applications.[1] The pore size, shape and dimensionality of zeolites are dependent not only on the framework cavities but also on the type of neutralizing cations contained in the nonframework structure. For instance, by exchanging the sodium cations in the A-type zeolite with the calcium cations, the