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Enzymatic Activity of Cellulase Adsorbed on Cellulose and Its Change During Hydrolysis

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ABSTRACT

Hydrolysis of pure cellulose Avicel has been carried out, using Meicelase from *Trichoderma viride*, where the enzymatic activity of cellulase adsorbed on cellulose and its changes during the hydrolysis were investigated. A rapid drop of the hydrolysis rate during the reaction, that is always observed in enzymatic hydrolysis of cellulose, could be explained by a decline of specific activity of adsorbed enzyme, and it was implied that the decline results from a loss of synergistic action between endoglucanase and exoglucanase. An empirical equation expresses the change of hydrolysis rate during the reaction and also shows that the change of the hydrolysis rate is caused by the decline of the specific enzymatic activity of adsorbed enzyme.

Index Entries: Enzymatic hydrolysis of cellulose; cellulase; kinetics; adsorption; Avicel.

NOMENCLATURE

E_{ads} concentration of adsorbed enzyme (mg-protein/mL)
Et total concentration of enzyme (mg-protein/mL)
k overall inhibition constant in Eq. (1) (dimensionless)

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m Parameter in Eq. (2) (dimensionless)
n parameter in Eq. (3) (dimensionless)
n_0 parameter in Eq. (4) (dimensionless)
q parameter in Eq. (4) (dimensionless)
S_0 initial concentration of cellulose (mg/mL)
t reaction time (h)
V hydrolysis rate of cellulose (mg/mL/h)
V_0 initial hydrolysis rate of cellulose (mg/mL/h)
X fractional conversion in the hydrolysis of cellulose (dimensionless)

INTRODUCTION

In the enzymatic hydrolysis of cellulose, it is well known that the reaction rate quickly declines in the early stage of the hydrolysis, even though the initial rate is large (*1*). The decrease in the hydrolysis rate obeys first-order kinetics vs the fractional conversion instead of the reaction time: namely

$$-d\,\mathrm{V} / d\,X = \mathrm{kV} \tag{1}$$

where V is the hydrolysis rate, X is the fractional conversion, and k is the overall inhibition constant (dimensionless) (*1*). In order to understand the decline, several changes during the hydrolysis have been investigated: for example, an increase in the product inhibition, a quick decrease in the surface area of cellulose accessible to cellulase (*2*), an increase in the crystallinity of cellulose (*1*), and so on. These investigations on the change in the hydrolysis rate have had some success. Converse and coworkers have attempted to explain the hydrolysis rate by the adsorption of cellulase on the substrate (*3*). We think that this is basically correct because the hydrolysis is heterogeneous. However, it is doubtful that the adsorbed enzyme always forms an active enzyme–substrate complex to release sugar from cellulose, and all of the adsorbed enzyme have the enzymatic activities. It is also doubtful that the activity is constant during the hydrolysis.

Cellulase is composed of many enzymes classified into three types, namely exoglucanase, endoglucanase, and β-glucosidase (*4*). In the hydrolysis of cellulose, the synergistic action of exoglucanase and endoglucanase plays a very important role to cut off sugar from cellulose (*4,5*). The hydrolysis rate depends on the ratio of the two component enzymes, and there is an optimal ratio to maximize the hydrolysis rate (*6*). In other words, the hydrolysis rate depends on how the component enzymes adsorb on the substrate. In some cases, the ratio of adsorbed exoglucanase and endoglucanase changes during the hydrolysis (*7*). It means that the enzymatic activity of adsorbed enzyme may change during the hydrolysis.

However, there are few reports on the subject. Converse has developed the kinetics by assuming the deactivation of cellulase adsorbed on the substrate (*3*). Nutor and Converse showed that, in the hydrolysis of pretreated poplar wood the hydrolysis rate per unit amount of adsorbed enzyme declines significantly as the conversion increases, and also showed that the specific rate at a given conversion is reduced by increased substrate concentration (*8*).

In this work, the hydrolytic activity of cellulase adsorbed on cellulose and its changes during the hydrolysis were investigated. We used cellulose powder Avicel as the substrate, because cellulase adsorbs on not only cellulose but also lignin (*9*) and the analysis of adsorption data must be complicated. We also tried to explain the empirical rate expression [Eq. (1)] from the activity of the adsorbed enzyme.

MATERIALS AND METHODS

Materials

Cellulase from *Trichoderma viride,* Meicelase CEPB-5029 (Filter paper activity: 8000 μ/mg), was supplied from Meiji Seika Kaisha Ltd., Japan and used without further purification. Cellulose powder Avicel was purchased from Ashahi Chemical Co., Japan. All other chemicals used were of reagent grade.

Enzymatic Hydrolysis

Avicel was placed in 50-mL screw-capped test tube and buffered to pH 4.8 with 0.1*M* acetate and the hydrolysis was started by adding cellulase solution. The initial concentrations of Avicel and cellulase were 5–10 mg/mL and 1.3 mg protein/mL, respectively. The test tube was sealed and incubated in shaking bath at 40°C. At given intervals, 0.6 mL sample of the suspension was withdrawn and centrifuged (5 min, 3000 rpm). The supernatant was used to determine the sugar concentration, the protein concentration, and the enzymatic activities of CMCase and β-glucosidase.

Determination of Sugar Concentration

Glucose concentration was determined using the glucose oxidase/peroxidase enzymatic assay reagent (Glucose B-test Wako; Wako Junyaku Kogyo). Total reducing sugar (TRS) was determined by dinitrosalicyic acid (DNS) method. TRS as glucose was determined from calibration curves for cellobiose and glucose. The conversion was calculated using the potential glucose.

Determinations of Protein Concentration and Enzymatic Activities of β-Glucosidase and CMCase in Liquid Phase During the Hydrolysis

Protein

The concentration of protein was determined by Bradford colorimetric assay from Bio-Rad Co., using bovine serum albumine as a standard.

β-Glucosidase

Fifty microliter of the supernatant was added to 3 mL of 0.33 mg/mL *p*-nitrophenylglycoside (PNPG) and incubated at pH 4.8 and 40°C. After 30-min incubation, the absorbance by *p*-nitrophenol (PNP) was measured at 400 nm.

CMCase

CMCase activity corresponding to endoglucanase activity was determined by viscosity. The supernatant withdrawn from the hydrolysis solution was diluted 10 times and 15 μL of the diluted solution was added to 15 mL of 0.5 wt% carboxymethyl cellulose (CMC) solution preheated to 40°C in an Ubelohde viscometer at pH 4.8. Change in the viscosity of the solution after 5-min incubation was measured.

Hydrolysis of Avicel in the Presence of Glucose

Glucose of 5–30 mg/mL working concentration were added to slurries of Avicel of 10–80 mg/mL and then the hydrolysis was started as described above. The sugar and protein in the suspension were measured after 1-h hydrolysis.

Preparation of Partially Prehydrolyzed Cellulose

Avicel (50 mg/mL) was prehydrolyzed by cellulase of 1.3 mg protein/mg at 40°C and pH 4.8. After given reaction time, the remaining cellulose was washed with distilled water and 1*M* NaCl, and then boiled in a water bath for 30 min, and washed again. The wet residue thus obtained were used as substrate in the hydrolysis by fresh enzyme. Care was taken for drying before the use.

RESULTS AND DISCUSSION

Figure 1 presents changes in the fractional conversion and the adsorbed enzyme concentration as a function of the reaction time in the hydrolysis of Avicel, respectively. The enzyme quickly adsorbs on the substrate and begins to return to the liquid phase as the hydrolysis proceeds. In general cases, the quickly adsorbed enzyme in the initial stage of the reaction monotonically returns to the liquid phase as the reaction proceeds (*10,11*).

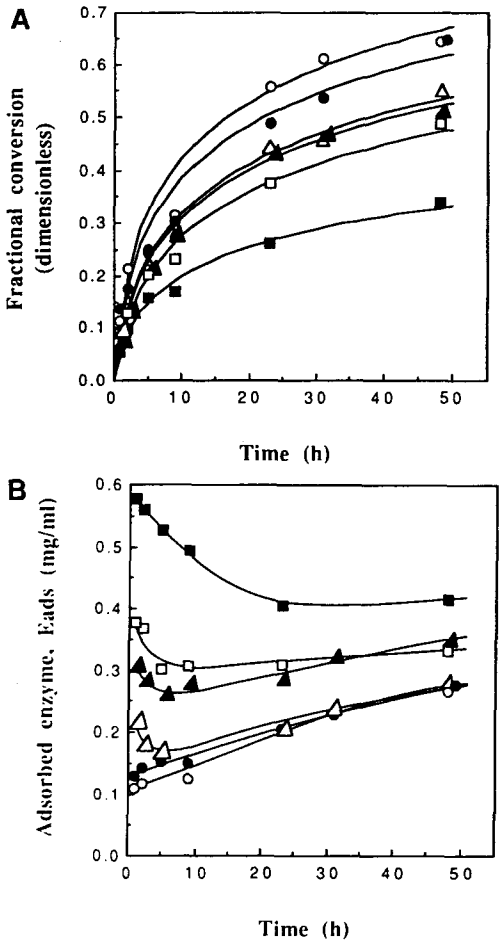

Fig. 1. **A:** Changes in fractional conversion of the hydrolysis of Avicel as a function of the reaction time. $E_0 = 1.3$ mg protein/mL. Solid curves were estimated by Eq. (7). (S_0 (mg/mL), V_0 (mg/mL·min)), k (dimensionless)); ((○) 6, 1.2, 6.2); ((●) 12, 2.1, 6.6); ((△) 24, 2.8, 6.9); ((▲) 36, 4.0, 7.0); ((□) 47, 4.3, 7.4); and ((■) 95, 7.1, 11.4). **B:** Changes in adsorbed enzyme concentration as a function of the reaction time. $E_0 = 1.3$ mg protein/mL. S_0 (mg/mL): 6 (○); 12 (●); 24 (△); 36 (▲); 47 (□); and 95 (■).

However, as Figure 1 shows, the desorption from Avicel was gradually returned to adsorption again. A similar result has been obtained by Matsuno et al. by using microcrystalline cellulose (MCC) as a substrate and cellulase from *E. javanicum* (*12*). Matsuno explained the adsorption by assuming that the enzyme adsorbs not only on the surface of the cellulose fibrils, but that it diffuses into the inside of the fibrils and adsorbs there also. Matsuno further claims that the difference in the adsorption pattern comes from the competition between the hydrolysis rate and the diffusion rate of enzyme: in the case that the reaction is fast, the adsorbed enzyme

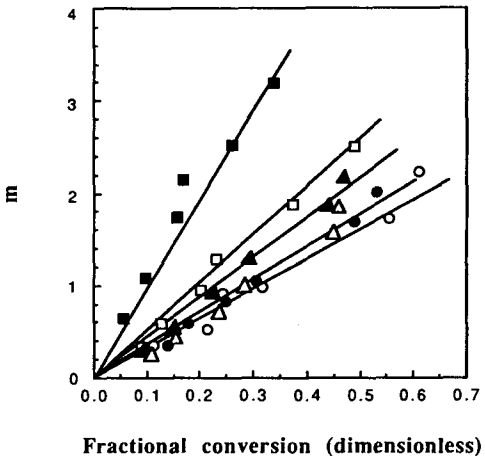

Fig. 2. Dependency of m-value in Eq. (2) on the fractional conversion. E_0=1.3 mg protein/mL. S_0 (mg/mL): 6 (○); 12 (●); 24 (△); 36 (▲); 47 (□); and 95 (■).

monotonically returns to the liquid phase. In addition to the explanation, we suppose that the structure of cellulose fibrils located in the outer shell of Avicel and MCC, which are prepared by acid treatment of lignocellulose, may be different from the structure of the inside: the outer shell may be plane and nonporous and the inside may be more porous. At any rate, if the hydrolysis rate is in proportion to the adsorbed enzyme concentration, it must follow the adsorption curve. The data shown in Fig. 1 A and B deny the proportional relationship.

We assumed that the relationship between the hydrolysis rate and the adsorbed enzyme concentration could be expressed by

$$V / V_0 = (E_{ads} / E_t)^m \quad (2)$$

where E_t is the total enzyme concentration, E_{ads} is the concentration of adsorbed enzyme, m is an arbitrary constant, and the subscript *"0"* means the initial value, and also assumed that the m value might change as the reaction proceeds. Figure 2 presents the m value vs the fractional conversion calculated from the relative reaction rate and the adsorbed enzyme concentration at given conversion in the hydrolysis shown in Fig. 1. Figure 2 shows that changes in the hydrolysis rate can be related to changes in the adsorbed enzyme concentration by assuming that the m value is in proportion to the fractional conversion. In other words, the relationship between the hydrolysis rate and the adsorbed enzyme concentration and its change during the reaction can be expressed as follows:

$$V / V_0 = (E_{ads} / E_t)^{nX} \quad (3)$$

where n is a constant: $n = m/X$. Equation (3) means that the hydrolysis rate is not in direct proportion to the adsorbed enzyme concentration, and

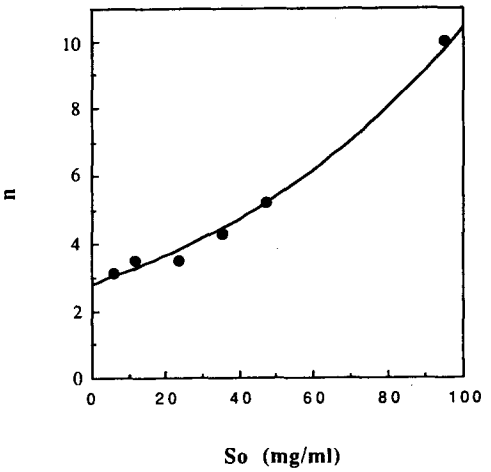

Fig. 3. Dependency of n-value in Eq. (3) on the initial substrate concentration, S_0. E_0=1.3 mg protein/mL. Solid curve: Eq. (4), where n_0=2.82 and $q=1.30\times10^{-2}$.

that the activity of the adsorbed enzyme decreases during the hydrolysis as Nutor et al. have reported (*8*).

Figure 3 presents the n value vs the initial substrate concentration S_0. The relationship can be expressed by

$$n = n_0 \exp(qS_0) \tag{4}$$

where n_0 and q are constant. In the present case, when S_0 is in mg/mL, n_0 and q were 2.82 and 1.30×10^{-2}, respectively. Equations (3) and (4) show that the higher the initial concentration of substrate, the faster the hydrolysis rate declines. Similar dependency of the decline on the initial substrate concentration has been obtained experimentally by Nutor et al.

By a differentiation of Eq. (3) against X, we obtain

$$-dV/dX = kV \tag{5}$$

where

$$k = -n\{ln\,(E_{ads}/E_t) + (X/E_{ads})d\,E_{ads}/d\,X\} \tag{6}$$

Equation (5) is exactly the same as Eq. (1). Equation (1) empirically obtained from the plot of ($-ln$ V) vs X was able to be derived from the decline in the activity of adsorbed enzyme during the hydrolysis. When Eq. (1) was obtained, the parameter k was assumed to be constant over the reaction (*1*). However, in the present work, it was found that the k value changes depending on the adsorbed enzyme concentration. The parameter k is constant only when the adsorbed enzyme concentration does not change, namely when $d\,E_{ads}/d\,X=0$. In this case, we obtain an integral formula of Eq. (3),

$$P = (S_0 / k)\, ln\, \{1 + (V_0 / S_0)kt\} \quad (7)$$

where

$$k = -\, n\, ln\, (E_{ads} / E_t) \quad (6')$$

and P is the concentration of the product. There have been some cases in which the adsorbed enzyme concentration does not so much change over the full range of the reaction, or at least in the early stage of the hydrolysis (*9*). However, in the more general cases, as described above, the enzyme quickly adsorbed in the earlier stage of the reaction and gradually desorbs from cellulose as the hydrolysis proceeds. In this case, the *k* value would change according to Eq. (6). Equation (1), and hence Eq. (7) in which k value is constant, has been applied to the hydrolysis of many kinds of substrates with some success (*8,13*). The solid curves in Fig. 1 (A) were computed from Eq. (7) using the least squares parameter values given in the legend of the figure. If the change in the adsorbed enzyme concentration during the hydrolysis had been taken account of in the *k* value, the accuracy in the estimation of *P* vs *t* would be better. In order to do so, changes in *Eads* during the hydrolysis must be formulated, and we will discuss this in another paper. The hydrolysis rate is not in direct proportion to the adsorbed enzyme concentration, and the activity of the adsorbed enzyme declines as the reaction proceeds.

Glucose Inhibition

Figure 4 A presents the effects of glucose on the adsorption of cellulase on Avicel. The adsorption was not affected by glucose. Similar result has been obtained by Ooshima et al., where it has been reported that cellobiose also does not affect the adsorption (*9*). Figure 4 B presents the initial hydrolysis rate per enzyme adsorbed on the substrate vs glucose added. It shows that the hydrolysis is inhibited by glucose as has been reported often. The degree of the inhibition depends on the glucose concentration, but seems not to depend on the initial concentration of the substrate. We can estimate the degree of the drop of the hydrolysis rate per adsorbed enzyme by a given concentration of glucose from Fig. 4 B. For example, in the case of the hydrolysis of 35.6 mg/mL Avicel shown in Fig. 1 A, the rate per adsorbed enzyme would drop to 70% of the initial one at 20% conversion, if the drop is attributed only by glucose. However, as shown in Fig. 1 B, the rate drop experimentally obtained was 44%. Other cases were compared in Table 1. From the behavior shown in Table 1, we conclude that the decline in the specific rate with conversion is not as a result of glucose inhibition, although it is true that glucose inhibition can cause decline.

Change in a Structure of Cellulose by Hydrolysis

The partially prehydrolyzed and then washed Avicel was hydrolyzed again by fresh enzyme of 1.3 mg protein/mL. Figure 5 presents the enzyme

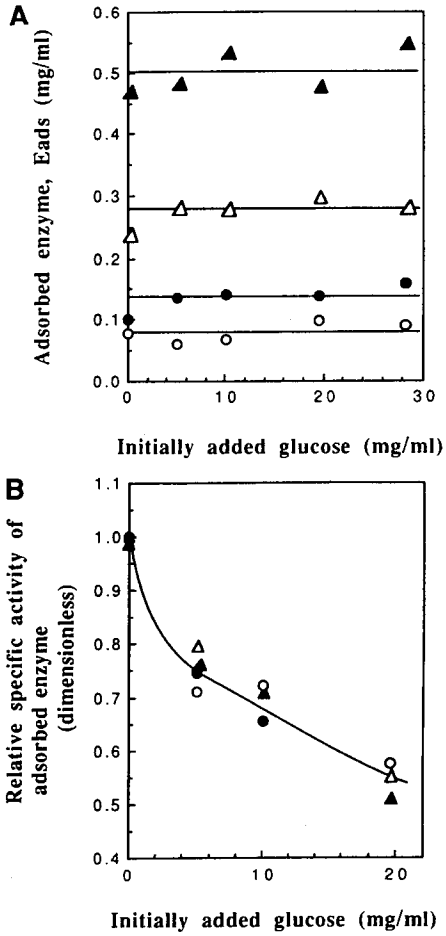

Fig. 4. **A:** Effect of glucose on adsorption of enzyme. E_0=1.3 mg protein/mL. S_0 (mg/mL): 10 (○); 20 (●); 40 (△); and 80 (▲). **B:** Effect of glucose on the specific activity of adsorbed enzyme. E_0=1.3 mg protein/mL. S_0 (mg/mL): 10 (○); 20 (●); 40 (△); and 80 (▲).

concentration adsorbed on the celluloses in the second hydrolysis of 60 min. The larger the conversion in the prehydrolysis, the smaller the newly adsorbed enzyme concentration. The decrease in the adsorbed enzyme concentration with the conversion of the prehydrolysis is attributable to decrease in the surface area of cellulose by the prehydrolysis as Burns et al. have been reported (2). Figure 5 B presents the initial hydrolysis rate per adsorbed enzyme in the second hydrolysis, where the initial hydrolysis rate was determined from the sugar concentration after 1 h reaction. The activity of the newly adsorbed enzyme was found to be almost same even though the substrates were different in the degree of the prehydrolysis. From this we conclude that the decline of the enzymatic activity of adsorbed enzyme shown in Fig. 2 is not caused by the structural change of cellulose by the hydrolysis.

Table 1
Decrease in Specific Activity of Adsorbed Enzyme by Glucose in the Hydrolysis of Avicel[a]

Initial substrate concentration mg/mL	Remaining specific activity of adsorbed enzyme at the fractional conversion 0.2	
	Estimated from Fig. 4 (b) %	Observed %
6	89	44
12	82	44
24	75	44
36	70	44
47	66	38
95	55	22

[a]Comparison between the activities estimated from Fig. 4 (b) and observed.

Deactivation of Cellulase

Incubation of enzyme at 40°C and pH 4.8 for 50 h did not influence the Avicelase activity at all, although the data are not shown here. This result implies that the enzyme in the liquid phase is not denatured during the hydrolysis. On the other hand, it is difficult to evaluate the stability of the adsorbed enzyme. Nevertheless, we doubt that the enzyme molecule adsorbed on cellulose itself is denatured during the hydrolysis, because the cellulolytic activity of the liquid phase is in proportion to the concentration of the protein, and a significant part of the activity should be attributed to the protein that, once adsorbed on cellulose, then comes back to the liquid phase again as the reaction proceeds (*9*).

Change in Adsorption Ratio of Endoglucanase and Exoglucanase

β-glucosidase did not adsorb on Avicel at the reaction temperature of 40°C as previously reported (*7,9*). Figure 6 presents both activities of CMCase and protein concentration in the liquid phase during the hydrolysis of 80 mg/mL Avicel. Initially adsorbed CMCase gradually returned to the liquid phase and the recovery to the liquid phase reached almost 100% by the fractional conversion of 0.5. However, 20% of proteins is still left adsorbed on cellulose. The enzyme remaining on cellulose is probably exoglucanase. Figure 6 shows that the ratio of endoglucanase (CMCase) and exoglucanase adsorbed on cellulose changes during the hydrolysis. It is well known as described above that endoglucanase and exoglucanase synergistically work for the hydrolysis of cellulose (*5*), and that there exists an optimal ratio between the two enzymes to maximize the hydrolysis rate. The change in the adsorption ratio shown in Fig. 6 must influence

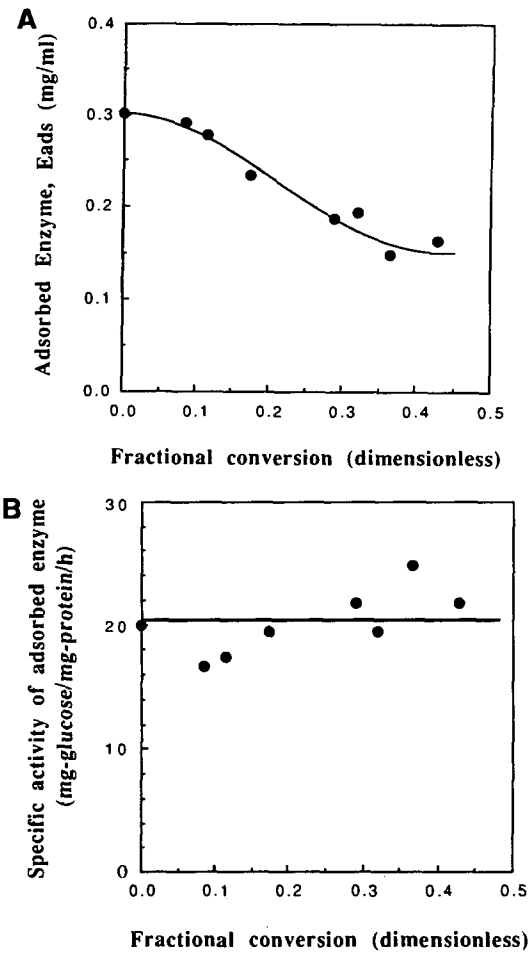

Fig. 5. **A:** Adsorption of enzyme on the partially prehydrolyzed Avicel. E_0=1.3 mg protein/mL. S_0=50 mg prehydrolyzed/mL. **B:** Specific activities of enzyme adsorbed the prehydrolyzed Avicel as a function of the fractional conversion in the prehydrolysis. E_0=1.3 mg protein/mL. S_0=50 mg prehydrolyzed/mL.

the synergistic action between endoglucanase and exoglucanase. The decline of the specific activity of the adsorbed enzyme, expressed by Eq. (3), is implied to be owing to the change of the synergistic effect. Matsuno (*12,14*) claims that the rapid decrease in the reaction rate, as shown in Fig. 1A, is caused by the diffusion of cellulase into the cellulose fibril, namely into the small pores (2) and as a result of that, the rate of the hydrolysis by the diffused enzyme is low. Since the hydrolysis rate is in proportion to pore volume accessible to cellulase (*2,15*), it is likely that the diffusion of cellulase into the pores is important in effective hydrolysis. Furthermore, it should be noted that it is important for both endoglucanase and exoglucanase to enter the pores, because their synergistic action is neces-

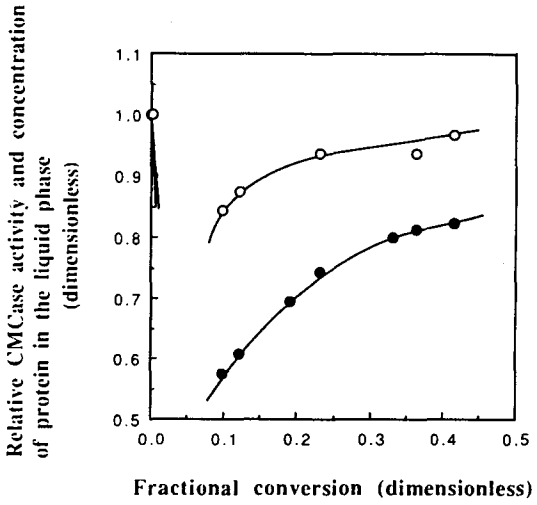

Fig. 6. Change in CMCase activity and protein concentration in the liquid phase as a function of the reaction time. E_0=1.3 mg protein/mL. S_0=80 mg/mL. CMCase(○) and protein (●).

sary for the effective and rapid hydrolysis of cellulose. Our present work implies that in very early stage of the reaction, the two enzymes must enter the pores together and then endoglucanase desorbs from the surface of cellulose and exoglucanase remains in pores. The separation and the localization of exoglucanase from endoglucanase must be disadvantageous to the reaction. The difference in the behavior of the two enzymes may be that exoglucanase adsorbs more strongly on cellulose than endoglucanase (7). According to Eqs. (3) and (4), the disadvantage in the synergistic action caused by the separation of endoglucanase from exoglucanase is probably more significant when the substrate concentration is large, that is, when the density of the adsorbed enzyme is low.

CONCLUSION

The hydrolysis of pure cellulose Avicel has been carried out, using Meicelase from *Trichoderma viride,* where we noted a rapid drop of the hydrolysis rate during the reaction and investigated the enzymatic activity of cellulase adsorbed on cellulose and its changes during the hydrolysis. As a result, the rapid drop of the hydrolysis rate with the progress of the reaction could be explained by a decline in enzymatic activity of cellulase adsorbed on cellulose, and it was implied that the decline is attributable to an occurence of disadvantage in synergistic action between endoglucanase and exoglucanase during the reaction. The details of the results are summarized as follows:

1. The specific activity of adsorbed enzyme (activity/adsorbed enzyme) declines during the hydrolysis and the decline can be expressed by Eqs. (2) and (3);
2. The higher the initial substrate concentration, the faster the decline becomes. The relationship is expressed by Eqs. (3) and (4);
3. By integrating Eq. (3), we obtained an empirical equation expressing the change of hydrolysis rate during the reaction and which also shows that the change of the hydrolysis rate is as a result of the decline of the specific activity of adsorbed enzyme. The equation was exactly the same as the equation [Eq. (1)] that was previously obtained by one of us, although in the previous equation the physical meaning of paramerer k was not clear;
4. The decline of the specific activity of adsorbed enzyme during the hydrolysis could be explained by a change in adsorption ratio of endoglucanase and exoglucanase, in other words, by an occurrence of disadvantage in synergistic action between the two enzymes during the hydrolysis. Product inhibition was another reason for the decline. Other two factors, namely a change in reactivity of cellulose, and deactivation of adsorbed enzyme, were not in relation.

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