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Kinetic Studies on Enzymatic Hydrolysis of Cellulose(Ⅱ) - Evaluation of Several Factors for Enzyme Adsorption and Initial Hydrolysis -

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섬유소 가수분해반응에 관한 연구(Ⅱ) - 효소흡착과 가수분해반응에 관여하는 여러인자의 영향 -

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ABSTRACT

Enzymatic cellulose hydrolysis depends on the several factors such as the structural features (CrI, particle size and surface area, etc.), the nature of cellulase enzyme system, the inhibitory effects of products, and enzyme deactivation. At the presence of products on the initial hydrolysis rate of cellulose, cellobiose has more severe inhibitory effect than glucose. Othewise, the inhibition effect of products for adsorbed enzyme is related to the glucose and cellobiose concentration hyperbolically. Enzyme deactivation of FPA and β-glucosidase were expressed by exponential decay profile.

INTRODUCTION

Since native cellulosic materials are water-insoluble solid substrate, the cellulose-cellulase system is heterogeneous, and the hydrolysis reaction involves several steps. Enz ymatic hydrolysis of cellulosic materials primarily depends on 1) the substrate multiplicity and the structural features of cellulose, 2) the nature and mechanism of cellulose-cellulase system, 3) the inhibitory effects of products, and 4) enzyme deactivation(18). Due to the difficulty involved and the complexity of cellulose-cellulase system, not enough attention has been given to the study of the kinetic modelling. Only a few scientific contributions reported recently have dealt with the kinetic modelling of the enz ymatic hydrolysis of cellulosic materials. Several kinetic models have been proposed, based on the product inhibition(9), enzyme deactivation(6), enzyme adsorption(8), the multiplicity of cellulose(3), and the substrate characteristics(9).

Fan *et al.* have studied the kinetic characteristics of this heterogeneous cellulose-cellulase system. Their works include the effect of the major structural features on the hydrolysis rate(4), a kinetic analysis of the initial reaction period, and a kinetic analysis of the extended hydrolysis times(14). It has been found that the kinetic characteristics of this heterogeneous cellulose-cellulase system are substantially different from the homogeneous enzyme catalyzed hydrolysis reaction.

In this study the effect of structural features of cellulose, product inhibition, and enzyme deactivation on enzymatic

cellulose hydrolysis and enzyme adsorption were examined by experiments. The objective of this study is to develop a comprehensive kinetic model based on our experimental observations.

MATERIALS AND METHODS

The experimental methods, conditions and materials for this study were same that described in our previous report (19).

Enzymatic Hydrolysis of Cellulose

All cellulosic samples were dried overnight at 80℃, and 0.25, 0.5, 1.0, 2.0, and 4 g substrate were suspended in 30ml of water and 20ml of 0.05M citrate buffer(pH 4.8). The cellulose suspensions were incubated in a shaker bath at 50℃ for 24 hr before the addition of enzyme solution to assure the hydration(12, 13). After preincubation 50ml of enzyme solutions(0.101, 0.062, and 0.0378 FPU / ml) which were preheated to 50℃ in a water bath were added and the reactions were carried out at 50℃ in a shaker at 200 rpm. The samples were taken at predetermined time intervals, rapidly centrifuged to remove solids. Aliquots of supernatants were tested for reducing sugar and protein concentration after appropriate dilution. The samples were refrigerated for further analysis. Initial hydrolysis was determined from the polynominal fitting of experimental data up to 12 hr run.

Enzymatic Hydrolysis of Cellobiose

Kinetic studies of β-glucosidase were carried out using β-D-cellobiose(Fluka chemie AG) as substrate(1-10 mg / ml). The other experimental procedures are similar to the case of enzymatic hydrolysis of cellulose. After preincubation of cellobiose solution(5ml), the enzyme solution (5ml) was added and the reaction was carried out at 50℃ in a shaker for 30 min at 200 rpm. The reaction was stopped by submersion in a boiling water bath for 5 min. β-glucosidase activity was measured by release of glucose in 30 min from mixture of 1 ml of crude enzyme and 1 ml of 20 mM cellobiose in citrate buffer(21) or was determined by similar method of Gao Peiji(16).

β-glucosidase activities measured were 0.08083, 0.05018, and 0.03549 EU / ml, respectively.

RESULTS AND DISCUSSION

Initial Hydrolysis Rate of Cellulose

Table 1. Change of initial reaction rate of fractionated and various cellulose

	Initial reaction rate (mg R.S / ml−hr)						
BW 40		control	60−120	120−170	170−200	200−270	270−325
	C_o(mg / ml)						
P_o=	40	2.1011	−	−	−	−	−
0.20685	20	1.2793	0.9503	0.9434	1.1370	1.2030	1.1520
mg pr / ml	10	0.8541	0.6224	0.6795	0.6878	0.7205	0.6755
C_o=	P_o(mg / ml)						
10.0	0.41	1.1509	−	−	1.3824	1.5700	1.7680
mg / ml	0.1034	0.4864	−	−	0.5910	0.6906	0.7127
Avicel		control	120−170	170−200	200−270	270−325	325−400
PH 101	C_o(mg / ml)						
P_o=	40	0.9631	−	−	−	−	−
0.20685	20	0.9575	0.4909	0.7908	0.9949	0.9489	1.1009
mg pr / ml	10	0.6258	0.3380	0.6460	0.6928	0.7641	0.8051
C_o=	P_o(mg / ml)						
10.0	0.41	0.759	−	1.0030	1.0400	1.0780	−
mg / ml	0.1034	0.470	−	0.3415	0.3787	0.4297	−

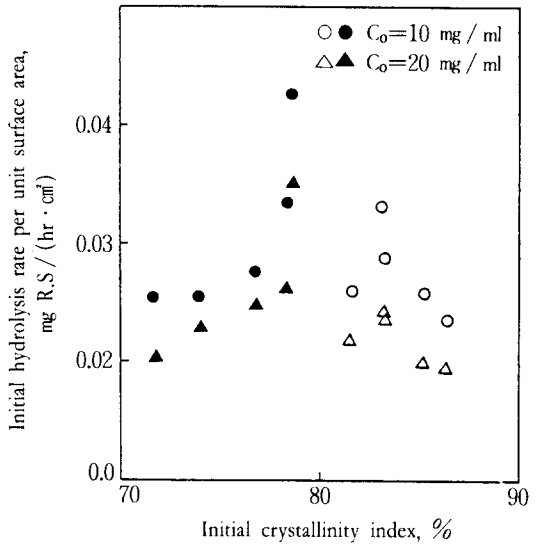

Fig. 1. Effect of crystallinity index on the initial hydrolysis rate at E_o=0.20685 mg pr / ml, (●▲) BW 40, (○△) Avicel PH 101.

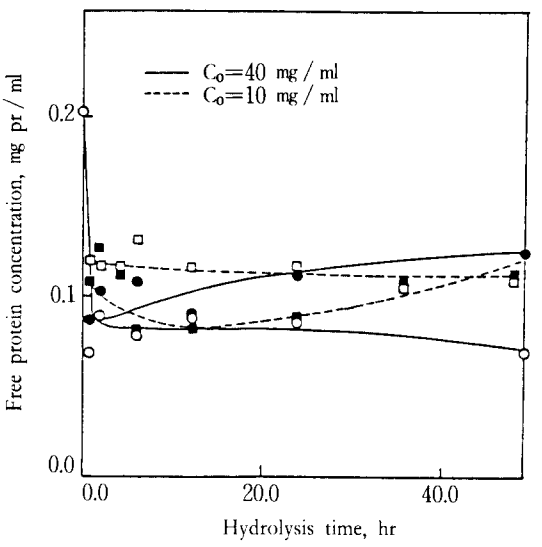

Fig. 2. Adsorption pattern during hydrolysis reaction of various cellulose at E_o= 0.20685 mg pr / ml, (○□) BW 40, (●■) Avicel PH 101.

The values of the initial apparent extent of soluble protein adsorption and initial apparent hydrolysis rate are listed in Table 1 to show their interdependence. The experimental data obtained from the fractionated and various cellulose sample at different soluble enzyme concentration. These results indicate that the initial hydrolysis rate depends strongly on the amount of adsorbed protein. The initial amount of adsorbed soluble protein depends on the initial cellulose concentration, enzyme concentration, and specific surface area of the cellulose. Also the initial reaction rate is not significantly increased as the particle size decreased. Fig. 1 shows that initial reaction rate per unit surface area of Solka Floc BW 40 and Avicel PH 101 have a distinct pattern for crystallinity. However, these results did not describe the overall hydrolysis reaction because the effect of product inhibition, enzyme deactivation and the other factors were not considered.

Adsorption Profile during Hydrolysis

As shown in Fig. 2, two distinctly different adsorption patterns during reaction were observed; one was a continuous increase in adsorbed protein during hydrolysis reaction(Solka Floc BW 40) and the other was a gradual release of enzyme protein from cellulose with the progress of reaction(Avicel PH 101).

By comparing the adsorption phenomena during reaction shown in Fig. 2, it was found that the enzymes were continuously adsorbed when the initial adsorption of enz ymes was restricted or hindered by an inaccessibility of substrates. When the enzymes were adsorbed to their maximum at the beginning of hydrolysis the enzyme proteins were gradually released with reaction time as the crystalline and inaccessible fraction of cellulose increased.

To explain the retardation of the reaction at the later phase, Lee and Fan have postulated that the surface area was composed of two fractions, active and inactive, and that the hydrolysis rate might be dependent on the active surface area, rather than on the overall surface area(15).

Change in Crystallinity during Hydrolysis

From the x-ray diffractograms it was found that the changes in crystallinity during hydrolysis reaction were closely related to the initial state of cellulose structure. As shown in Figs. 3–6, each of the crystallinity index of Solka Floc BW 40 and Avicel PH 101 gradually increased from 77.24 to 86.69 and from 84.0 to 88.7 during

48 hr of hydrolysis. The crystallinity index of Avicel PH 101 gradually increased but rather modestly. Caufield and Moore reported a similar increase in CrI during hydrolysis (2, 13). They concluded that the overall increase in digestibility was apparently a result of decreased particle size and increased available surface rather than a result of reduced crystallinity. Otherwise, Fan *et. al.* founded that the hydrolysis rate was mainly dependent upon the crystallinity rather than the simple surface area(4).

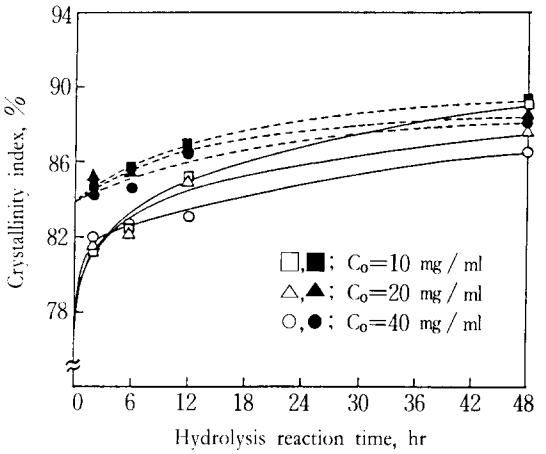

Fig. 3. Changes in crystallinity index during hydrolysis reaction at E_o=0.20685 mg pr / ml, (○△□) BW 40, (●▲■) Avicel PH 101.

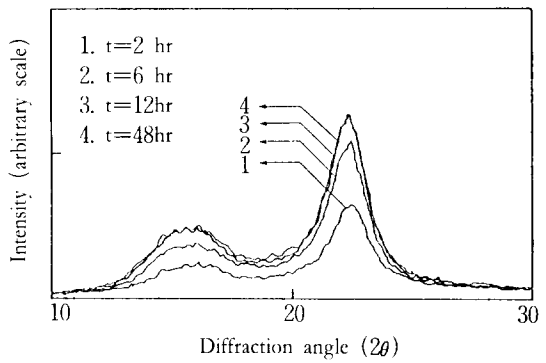

Fig. 4. X-ray diffractograms of Solka Floc BW 40 during hydrolysis at E_o=0.20685 mg pr / ml, and C_o=40 mg / ml.

This initial large increase in the crystallinity index for Solka Floc BW 40 indicated that the amorphous portion of cellulose was hydrolyzed more quickly than the crystalline portion. For highly crystalline cellulose such as Avicel PH 101, the hydrolysis rate dropped more significantly than amorphous cellulose. Since the increase in crystallinity during hydrolysis was not so great, the other factors(accessibility of cellulose surface, product inhibition, enzyme

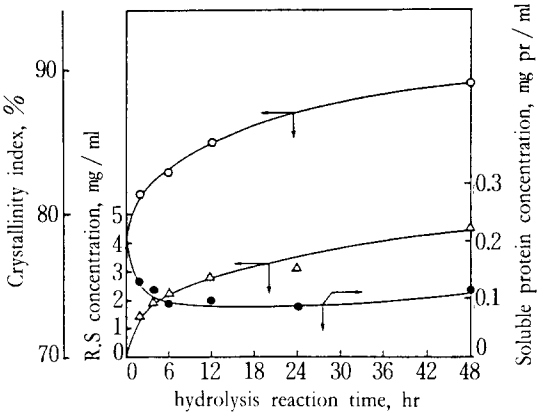

Fig. 5. Changes in structural parameters during hydrolysis reaction of Solka Floc BW 40 at E_o=0.20685 mg pr / ml, and C_o=10 mg / ml.

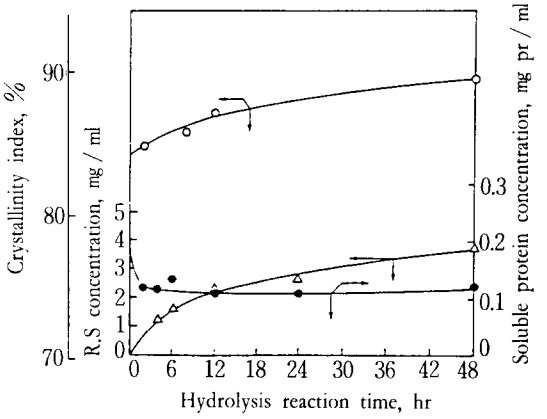

Fig. 6. Changes in structural parameters during hydrolysis reaction of Avicel PH 101 at E_o=0.20685 mg pr / ml, and C_o=10 mg / ml.

deactivation, etc.) might play a role in falling off the rate in later stage of the hydrolysis of crystalline cellulose.

Evaluation of Other Factors that affect the Hydrolysis Reaction

Product Inhibition

To determine the effects of the presence of products on the initial hydrolysis rate, experiments were carried out by adding 1, 2.5, 5, and 10mg of glucose or cellobiose to 10mg / ml of cellulose suspension at the onset of each experiment. The changes in adsorbed soluble protein and initial hydrolysis rate are presented in Fig. 7.

The reducing sugar concentration was obtained by subtracting the initailly added sugar from the reducing sugar concentration in the reaction mixture during 1 hr. The results show that the initial hydrolysis rate and adsorbed soluble protein decreased substantially. It appears that cellobiose has more severe inhibitory effect for initial hydrolysis rate than glucose. The results obtained by Lee *et al.*(14) are similar to the results obtained in the present work. Otherwise, glucose and cellobiose have been a similar inhibitiory effect on the adsorption process.

As the cellobiose concentration increased the extent of adsorption gradually decreased. It is therefore expected that the formation of cellobiose and deactivation during hydrolysis reaction can affect the adsorption and thereby reducing the amount of adsorbed enzymes. As a consequence, the fall-off in hydrolysis rate during the initial hydrolysis of crystalline and amorphous cellulose may be, in large part, attributed to the inhibition effect of product.

Enzyme Deactivation

Enzyme deactivation has been recognized as a serious problem in practical application of enzymatic hydrolysis of cellulosics. Typically the cellulase(based on CMC activity) of *Trichoderma* were reported to be remarkably stable and resistant to the inhibitor and other toxic compounds. More recent studies have shown that the exoglucanase activity is less stable(17).

To examine the enzyme stability, all stability experiments were carried out under the conditions employed for the enzymatic hydrolysis reaction(pH 4.8, 50℃, 200 rpm) without cellulose. It was found that both FPA and β-glucosidase activity were deactivated. Figs. 8 and 9 show that relative activity of enzyme protein during incubation time changes in a similar manner to the exponential decay profile.

About 60% of filter-paper activity and β-glucosidase

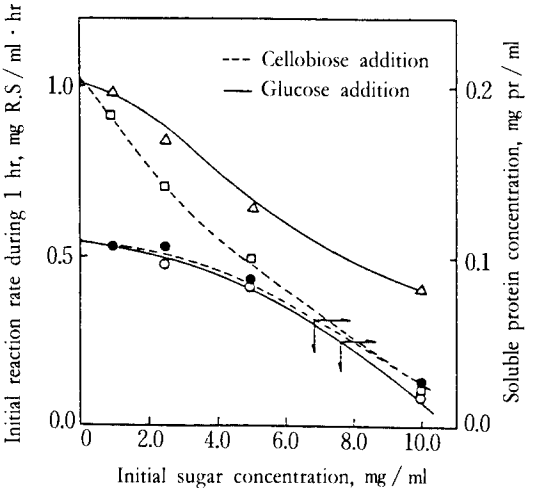

Fig. 7. Effect of product inhibition on the initial reaction rate and adsorption at E_o=0.20685 mg pr / ml, and C_o=10 mg / ml, BW 40.

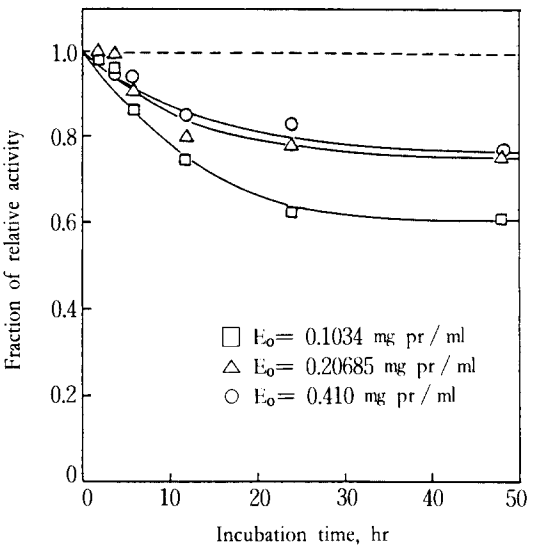

Fig. 8. Enzyme deactivation profile during incubation for FPA.

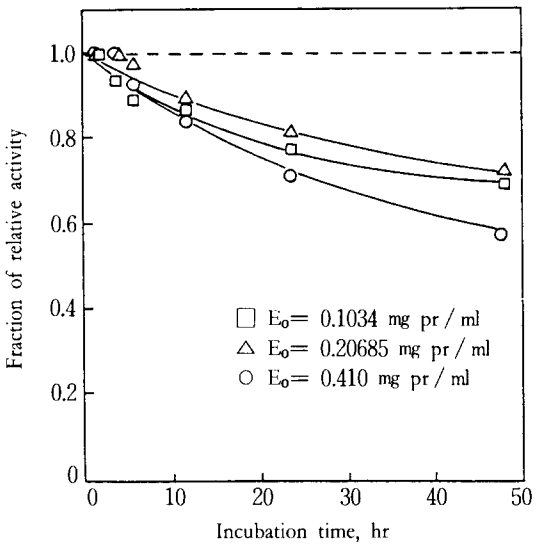

Fig. 9. Enzyme deactivation profile during incubation for β-glucosidase activity.

activity for low enzyme protein concentration(0.1034−0.41 mg pr / ml) were lost during 48 hr incubation. As the enzyme concentration increased, the extent of its deactivation slightly decreased. The possible mechanism of cellulase deactivation due to shaking or shearing action and thermal deactivation was described elsewhere(1, 10, 11, 17, 18).

Based on the results obtained from the experimental data, the effective enzyme activity for FPA and β-glucosidase can be expressed as follows;

$$[E_o]_{eff} / [E_o] = \theta_d(t) = Exp(-K_d\ t) \quad (1)$$

$$[E_{Bo}]_{eff} / [E_{Bo}] = \theta_{dB}(t) = Exp(-K_{dB}\ t) \quad (2)$$

The deactivation constant values are $k_d = 1.01 \times 10^{-2}$ hr^{-1}, $k_{dB} = 6.2716 \times 10^{-3}$ hr^{-1} for $P_o = 0.20685$ mg pr / ml, respectively. The values have been obtained from a deactivation study measuring total enzyme activity for FPA and β-glucosidase.

Initial hydrolysis of cellobiose

The initial reaction rate of cellobiose by β-glucosidase is plotted against the initial cellobiose concentration in Fig. 10. The initial rate was determined from glucose concentration after 30 min reaction time. Fig. 10 shows that the initial rate of glucose formation increases with increasing enzyme concentration and cellobiose concentration. At low

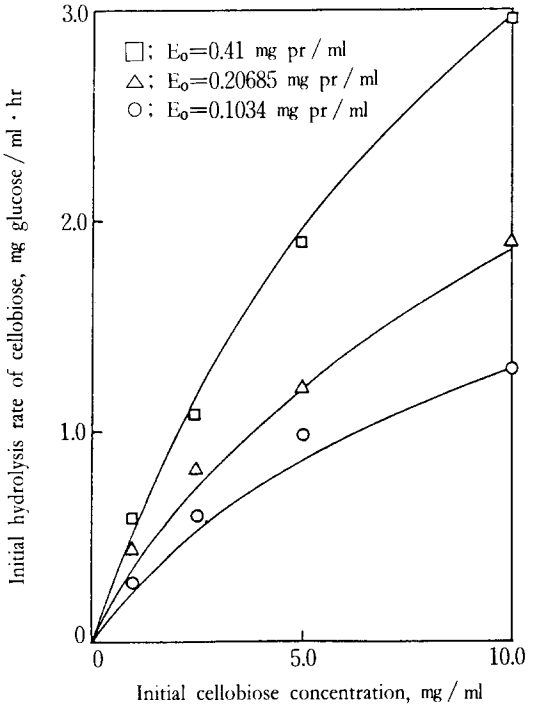

Fig. 10. Changes in initial cellobiose hydrolysis rate with crude enzyme.

enzyme concentration(E_{Bo}= 0.1034 mg pr / ml, 0.03549 EU / ml) initial glucose formation rate slightly increases with increasing substrate concentration(5−10 mg / ml). There is no cellobiose substrate inhibition effect as shown in Fig. 10. Grous *et. al.*(5) and Sundstrom *et al.*(20) observed similar results. While Hong et al.(7) observed substrate inhibition and from initial rate data developed a six-parameter model which tended to predict substantially higher conversions than observed for same reaction time. Therefore, the inhibition pattern may be varied by β-glucosidase characteristics isolated from each crude enzyme.

The effect of the cellobiose substrate inhibition on the initial hydrolysis rate of cellobiose may not be significant due to relatively high β-glucosidase activity of crude enz yme.

Enzymatic cellulose hydrolysis was dependent on the several factors such as the structural features of substrate, the nature of enzyme, the product inhibition, and the enz yme deactivation.

The reduction in the hydrolysis rate and enzyme adso-

rption is not due to the lack of substrate and enzyme deactivation, but rather to other cause, such as lack of accessible active site or transformation of cellulose into a form which is highly resistant to enzymatic attack and product inhibition.

요 약

섬유소 물질을 유용한 자원으로 전환시키기 위하여 섬유소 분해효소를 사용하여 가수분해 반응을 시킬 때 섬유소의 구조적 특성, 섬유소-효소계의 상관성, 가수분해 저해효과, 섬유소 분해효소의 불활성화 등에 의하여 섬유소 가수분해 반응 속도에 상당한 영향을 주는 것으로 알려져 있다. 가수분해 반응이 진행함에 따라 높은 결정성을 가진 섬유소인 Avicel PH 101의 결정화도는 서서히 증가하였으며 무정형이 많은 Solka Floc BW 40은 반응초기에 급격한 증가를 보여주었고 실험적으로 결정성 부분과 무결정성 부분이 동시에 분해됨을 알 수 있었으며, 반응 초기에 급격한 효소의 흡착이 일어나며 반응이 진행함에 따라 서서히 탈착됨을 볼 수 있었다. 반응 생성물인 글루코오스와 셀로바이오스는 초기 가수분해 반응속도에 상당한 저해효과를 주며 또한 생성물의 농도가 증가할수록 효소 흡착량이 감소함을 알 수 있었다. 섬유소 분해효소의 불활성도는 반응시간에 따라 지수 함수적으로 변화함을 실험적으로 알 수 있었다. 셀로바이오스의 초기 가수분해 반응속도는 상대적으로 높은 β-glucosidase의 활성도에 의해 비교적 높은 셀로바이오스의 농도(5-10mg/ml)에서도 기질의 저해효과가 적음을 볼 수 있었다.

NOMENCLATURE

C_o: Initial cellulose concentration (mg/ml)
CrI_o: Initial crystallinity index (%)
$E_{B,eff}$: Effective enzyme activity for β-glucosidase (EU/ml)
E_{Bo}: Initial β-glucosidase activity (EU/ml)
E_{eff}: Effective enzyme activity for FPA (FPU/ml)
E_o: Initial enzyme activity (FPU/ml)
$E_{o,eff}$: Effective enzyme activity for FPA (FPU/ml)
K_d: Deactivation constant for FPA (hr^{-1})
K_{dB}: Deactivation constant for β-glucosidase activity (hr^{-1})

GREEK LETTERS

θ_d: Relative fraction of deactivated FPA
θ_{dB}: Relative fraction of deactivated β-glucosidase activity

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