Kinetic Mechanism of Cellulose Hydrolysis by Endoglucanase I and Exoglucanase II Purified from *Trichoderma viride*

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A kinetic study of the hydrolysis of celluloses with different crystallinity by Endo I and Exo II purified from *Trichoderma viride* cellulase was performed. It was found that the hydrolysis of the celluloses can be described by a summation of two parallel pseudo first order reactions. From the experimental data, it is possible to calculate the fractions of easily and difficult hydrolyzable cellulose in Sigmacell 100 and associated rate constants. As the results, all of the rate constants decreased with increasing crystallinity of cellulose. From the kinetic data, it was also found that the coexistence of Endo I and Exo II showed a synergistic effect in the latter phase of the enzymatic hydrolysis reaction.

Introduction

The enzymatic degradation of cellulosic materials is catalyzed by three major components of the cellulase complex: endoglucanase, exoglucanase, and β-glucosidase. These cellulase components are often referred to as C_x, C_1, and cellobiase, respectively. The optimum pH and temperature of these enzymes are about pH 4.8 and 40 to 50 ℃, respectively, for *Trichoderma viride*.[1,2] The endoglucanase (Endo) and the exoglucanase (Exo) are partly known to act synergistically together in cellulose hydrolysis.[3] But it is difficult to completely understand the enzymatic hydrolysis of cellulose from theoretical approach and to develop its kinetics even for pure cellulose because the complex and enzymatic actions of multicomponent cellulase are complicated.[4~6] The mechanism and the kinetics of the enzymatic hydrolysis of cellulose are still not completely understood, and perhaps remains the most difficult problem in the enzymatic degradation of insoluble cellulosic materials. The difficulties in studying enzymatic hydrolysis of cellulosic materials are attributable to the complex properties of cellulose and its constituents as well as the multiplicity and complexity of the cellulase enzyme system.[6,7] The cellulase components act synergistically to affect hydrolysis of cellulose, and since the native material is insoluble in water solution, it is clear that one or more of the enzymes in question must adsorb to the cellulosic substrate so as to initiate the hydrolysis process.[5~7]

The physicochemical properties of the substrate,[6,8~10] the multiplicity of the cellulase complex,[11~13] as well as physical reaction parameters such as mass transfer[14] and temperature[10] have strongly influence on the cellulase adsorption. It has been reported that the relationship between free and adsorbed enzyme protein can be described by a Langmuir isotherm.[15]

Several investigations are recently suggested that the synergistic action of the cellulase components must be regarded as a phenomenon that is related to the competitive adsorption among the cellulase components, and have supposed that for hydrolysis of cellulose, the optimum initial ratio of endoglucanase to exoglucanase is greatly affected on the adsorption behavior of these enzymes.[1,11,16~19] Previously, we have described the adsorption behavior of Endo and Exo type cellulases partially purified from *Trichoderma viride* on microcrystalline cellulose. It was found that the maximal synergistic degradation occurs at the specific weight ratio of cellulase components at which the maximal affinity of cellulase components obtains.[16]

Various kinetic models have been developed to elucidate the enzymatic hydrolysis of cellulosic and lignocellulosic materials. Some of them are based on the assumption that the reaction rate is proportional to the amount adsorbed enzyme on the cellulose surface.[4] The models of Fan *et al.*,[20] and Grethlein[21] are based on the structural features of the substrate, like pore size distribution, crystallinity index, and specific surface area. Other model is based on the properties of the cellulase enzyme and the mass transfer in the reaction system.[22] Some theoretical derivations of a hydrolysis model are proposed.[23,24] Some authors, on the other hand, mathematical models to describe cellulase-cellulose adsorption based on the modified Langmuir type Equations have been derived.[25~27] Semiempirical models based on the assumption that the enzymatic reaction between cellulase and cellulose can be described by a summation of pseudo first order reactions have been developed.[28~31] A distributed parameter model proposed by Lee *et al.*[23] should take account of the various factors influencing the reaction rate such as structural properties of the substrate, adsorption-desorption phenomena of cellulase-cellulose system, mode of action of the cellulase, as well as mass transfer in the reaction system.[20,27,30] Kinetic model of the full time course of hydrolysis including enzyme adsorption has been developed by Lee and Fan,[32] Holtzapple *et al.*,[33] and Converse *et al.*.[34,35] All of these models assume that the initial rate of hydrolysis is proportional to the enzyme-substrate complex formed by adsorption of cellulase. However, another hypotheses have been postulated to explain the rapid decrease of hydrolysis rate already at a rather low conversion of the substrate. The main reason for this rate-retarding effect is still far from being completely understood, and has been diversely assumed to be related to thermal instability of the cellulases,[36,37] inactivation of the adsorbed cellulase due to the diffusion into the cellulose fibrils,[9,34,38]

strong inhibition by products cellobiose and or glucose,[32,39] transformation of the cellulose into a less digestible form,[32] and the heterogeneous structure of the substrate itself.[30,37,40] On the basis of a two-substrate hypothesis, mathematical models to describe enzyme adsorption and the kinetics of cellulose hydrolysis have been derived by Nidetzky and Steiner.[41] On the other hand, Converse and Optekar[35] have demonstrated that a two-enzyme component synergistic model can account for the observation that the degree of synergism goes through a maximum as the total enzyme concentration is increased. The degree of synergism declines at high enzyme concentration due to saturation of the adsorption sites with cellobiohydrolase, thus decreasing the generation of chain ends by endoglucanase.[35]

The presence of two different types of substrate in cellulose which differ in their susceptibility for enzymatic attack was proposed by Sattler *et al.*,[30] Gonzalez *et al.*,[37] and Wald *et al.*.[40] This idea led to the formulation of a relatively simple mathematical model which was successfully used to predict the hydrolysis kinetics of different substrates such as microcrystalline cellulose,[30,31] rice straw,[40] and wheat straw.[37] The assumption that amorphous and crystalline parts of the cellulose could account for the different reaction rates observed hydrolysis kinetics[40] has been contradicted by Ohmine *et al.*[42] and more recently by Lenz *et al.*.[43]

The object of this study is to demonstrate that the kinetic model for the enzymatic hydrolysis of cellulose is capable of representing synergistic behavior between Endo I and Exo II purified from *Trichoderma viride* by different crystallinity of celluloses. The effect of structural features of cellulose on cellulase adsorption and synergistic action between cellulase components was also investigated.

Materials and Methods

Materials. The cellulase enzyme, Meicelase TP 60 (Lot No. CEPB-5291), a commercial cellulase preparation of *Trichoderma viride* origin used in this study was kindly provided by Meiji Seika Kaisha Ltd., Tokyo, Japan. The substrate for the hydrolysis was microcrystalline cellulose, Sigmacell 100 (particle size, 100 μ, Sigma Co., USA). Other substrates for the determination of enzyme activities were Avicel pH 101 (microcrystalline cellulose, FMC Co., USA), carboxymethylcellulose (CMC, medium viscosity, Sigma Co., USA), and p nitrophenyl β-D glucopyranoside (PNPG, Sigma Co., USA). Packed column materials were Bio-Gel P 10 (100-200 mesh), Bio-Gel P 100 (100-200 mesh), DEAE-Bio-Gel A (Bio-Rad Laboratories, Richimond, USA), and SP-Sephadex C 50 (Pharmarcia Fine Chemicals, Uppsala, Sweden). All other reagents used were of analytical grade.

Enzymes. Enzyme activities towards Avicel pH 101, CM-cellulose, H_3PO_4-treated cellulose, and p-nitrophenyl β-D-glucopyranoside were measured as described by Kim *et al.*,[44] Somogyi,[45] and Shoemaker and Brown.[46] Major cellulase components, such as endoglucanase I (Endo I) and exoglucanase II (Exo II) were isolated from a commercial cellulase (Meicelase TP 60) derived from the fungus, *Trichoderama viride* by a series of chromatography involving Bio-Gel P 10, Bio-Gel P 100, DEAE-Bio-Gel A, SP-Sephadex C 50, and Avicel pH 101.[44] The purified Endo I and Exo II showed a single band on SDS-polyacrylamide gel electrophoresis. The average molecular weights determined by SDS- polyacrylamide electrophoretic analysis were 52,000 and 62,000 for Endo I and Exo II, respectively.[44]

Pretreatment. The H_3PO_4-treated Sigmacell 100 was prepared using 85% phosphoric acid. The prewetted Sigmacell 100 was mixed with H_3PO_4 solution and was allowed to stand for 1 day at 2 ℃ with cooling. The treated Sigmacell 100 was regenerated or precipitated by adding sodium hydroxide solution. This was washed with distilled water until the wash water was neutral.[6,47] Sigmacell 100 was pretreated with cellulase in 0.1 M sodium acetate buffer solution at pH 5.0. Ten g of Sigmacell 100 was accurately weighed, immersed in 150 mL enzyme buffer solution at pH 5.0, and incubated at 50 ℃ with shaking at 120 strokes/min. The final enzyme concentration was 0.67 mg/mL. The reaction was stopped after 12 and 48 hr, respectively. The Sigmacell 100 residue was filtered and washed successively with 100 mL distilled water, 900 mL 1.0 M NaCl solution, and 800 mL 0.1 M sodium acetate buffer solution at pH 5.0. After freezed drying, this pretreated cellulose was then used as substrate further for the hydrolysis.

Hydrolysis. Sigmacell 100 and pretreated Sigmacell 100 having different crystallinity were used as substrates for the hydrolysis. A 25 mg of substrate was accurately weighed, immersed in 2 mL of 0.05 M sodium acetate buffer solution at pH 5.0 containing corresponding enzyme, and incubated at 50 ℃ with shaking at 120 strokes/min. The final enzyme concentration was 2 mg/mL. The reaction was stopped at a desired time. The concentrations of glucose and cellobiose contained in the supernatant were then quantitatively determined by HPLC (Waters Model 401 unit, Waters Associates, Inc., Milford Mass. USA) using a Waters carbohydrate analysis column μ-Bandar-Pak, C_{18} Guard-Pak, and cation guard column as described by Kim *et al.*,[47] and or the DNS method.[44,45] The amount of enzyme in the supernatant was determined by the Lowry method[48] using bovine serum albumin as a standard.

Crystallinity. The crystallinity of the substrates was measured by the powder method of X-ray diffraction using a X-ray diffraction analyzer (Rikakikai Co., Japan).[8,31] The specimen was mounted horizontally while the Geiger counter moved in a vertical arc. A Cu-Kα target with a nickel filter was used at 35 kV of tube potential and 20 mA. The substrate sample was dried overnight at 80 ℃ and stored in a desiccator. Care was exercised in handling the samples to minimize exposure to the atmosphere because adsorption of moisture from the air tends to increase the crystallinity index.[20] The specimen was prepared by the mothod of McCreery.[49] The samples were scanned for a range of 2θ from 10° to 36°. The crystallinity index (CrI) was obtained by the following relatinship proposed by Segal *et al.*[50] was employed:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (1)$$

where I_{002} is the intensity of the 002 peak at about $2\theta = 22°$, and I_{am} is the intensity at $2\theta = 18°$. The I_{002} peak corresponds to the crystalline fraction and the I_{am} intensity corresponds to the amorphous fraction. From X-ray diffractograms, the relative correlation crystallinity index (C_c) was obtained by the following Equation:[51]

$$C_c=\frac{I_{ss}(\theta)-I_{am}(\theta)}{I_{cr}(\theta)-I_{am}(\theta)} \quad (2)$$

where $I_{ss}(\theta)$ is the X-ray diffraction intensity of each cellulose sample, and $I_{am}(\theta)$ and $I_{cr}(\theta)$ are the intensities of H_3PO_4-treated Sigmacell 100 and highly ordered 48 hr pretreated Sigmacell 100 as standard celluloses, respectively. The correlation crystallinity index may be found from the slope of the plot $\{I_{ss}(\theta)-I_{am}(\theta)\}$ versus $\{I_{cr}(\theta)-I_{am}(\theta)\}$ for 2θ from 10° to 36°.

Results and Discussion

A cellulose contains crystalline and amorphous regions, and the crystallinity is a measure of the relative amounts of these two regions. The crystallinities of various cellulosic substrates were measured in this work using a X-ray diffraction analyzer. The X-ray diffractogram of various cellulosic substrates showed that they had a different crystallinity index and intensities at the 2θ decreased in parallel with decrease in the crystallinity, is shown in Figure 1. The value of the relative crystallinity index (C_c) of each cellulosic substrate was determined for 2θ from 10° to 36° based on the C_c values of 48 hr pretreated Sigmacell 100 and the H_3PO_4-treated Sigmacell 100 taken as 1 and 0, respectively.[51] The C_c values of the H_3PO_4-treated Sigmacell 100, Sigmacell 100, and 12 hr pretreated Sigmacell 100 were 0, 0.73 and 0.84, respectively, while 48 hr pretreated Sigmacell 100 had a C_c value of 1. These increase in the correlation crystallinity index indicated that the amorphous portion of Sigmacell 100 was hydrolyzed more quickly than the crystalline portion.[20] Caulfield and Moore[52] indicated that the amorphous portion is hydrolyzed at about twice the rate of the crystalline portion.

The relationship between the extent of hydrolysis of substrate and reaction time over a period of 80 hr as measured with diffrent enzyme-substrate conditions is shown in Figure 2. For all enzyme-substrate mixtures, an initial fast reaction running about 12 hr is followed by a slow reaction. As shown in Figure 2, both Endo I and Exo II produced large amount of reducing sugar from less crystalline cellulose such as unpretreated Sigmacell 100, than those of pretreated Sigmacell 100 celluloses. On the other hand, Exo II in combination with Endo I produced larger amount of reducing sugar than that of Endo I and or Exo II alone in the hydrolysis. The curves in Figure 2(A) and (B) show the glucan amount of the conversions for Endo I and Exo II alone. But the curves in Figure 2(C) show that Exo II in combination with Endo I resulted in a synergistic effect. As shown in Figure 2, the amount of reducing sugar of the conversions for the combination of Endo I and Exo II is larger than that of the mathematical sum of those for Endo I and Exo II alone. These phenomena are due to the synergism of Exo II in combination with Endo I. Exo II produced lesser amount of reducing sugar than that of Endo I cellulase. These phenomena indicated that the extent of hydrolysis depended highly upon the structural properties of the cellulose, mode of action of the cellulase, and the multiplicity of the cellulase complex. In spite of the many studies, however, the principles and general kinetics for the production of sugar in the enzymatic hydrolysis of cellulose are still under study, due to the multiplicity of the structural characteristics of the substrate and the complexity of the cellulase enzyme system.[7] Therefore, a study on the rate and kinetics of enzymatic hydrolysis of cellulose is very important. In Figure 3, the semilogarithmic plots of ln C versus reaction time of the hydrolysis are presented. These support that the kinetic has a twophasic behavior which can be described by two parallel first order reactions. This confirms several reported experimental data,[29] and is consistent with Brandt *et al.*,[28] Sattler *et al.*,[30] and Kim *et al.*.[31] They have proposed that the cellulose hydrolysis can be described by a summation of two parallel first order reactions. Our experimental data show that two parallel first order reactions are sufficient to describe the Sigmacell 100 cellulose hydrolysis to conversion according to Equation (3):

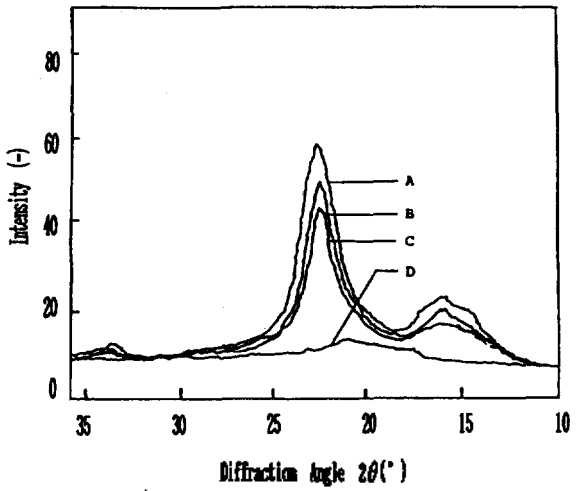

Figure 1. X-ray diffractogram of microcrystalline cellulose, Sigmacell 100. A; 48 hr pretreated Sigmacell 100, B; 12 hr pretreated Sigmacell 100, C; Sigmacell 100, D; The H_3PO_4-treated Sigmacell 100.

$$C_t=C_a\ \exp(-k_a\cdot t)+C_b\ \exp(-k_b\cdot t) \quad (3)$$

where C_t is the residual concentration of cellulose in g/L, and C_a and C_b are concentrations of the easily (C_a) and the difficult (C_b) hydrolyzable portion of the cellulose in g/L. Further, k_a and k_b are rate constants (h^{-1}) associated with C_a and C_b. The symbol of t respresents hydrolysis time. In the experiment, one measures the concentration of the solubilized reducing sugars at different times from which the concentraton of cellulose hydrolyzed ($Y=(C_a+C_b)-C_t$) can be calculated. We know that only for cellulosic substrates consisting of 100% glucan, the sum of C_a+C_b is identical to the initial substrate concentration. For the Sigmacell 100 used in our experiment, we have estimated the glucose content and found on average 98% glucan and traces of mannans. Therefore, under this experimental condition, the ratio $(C_a+C_b)/C_o=1$. The amount of Y/C_o is given by

$$\frac{Y}{C_o}=1-\frac{C_a}{C_o}\exp(-k_a\cdot t)-\frac{C_b}{C_o}\exp(-k_b\cdot t) \quad (4)$$

where the ratio Y/C_o is the fraction of hydrolyzed cellulose in g/g initial substrate at time t, and C_a/C_o and C_b/C_o give the fraction of the two types of cellulose in the substrate used. This Equation should be valid for all cases in which

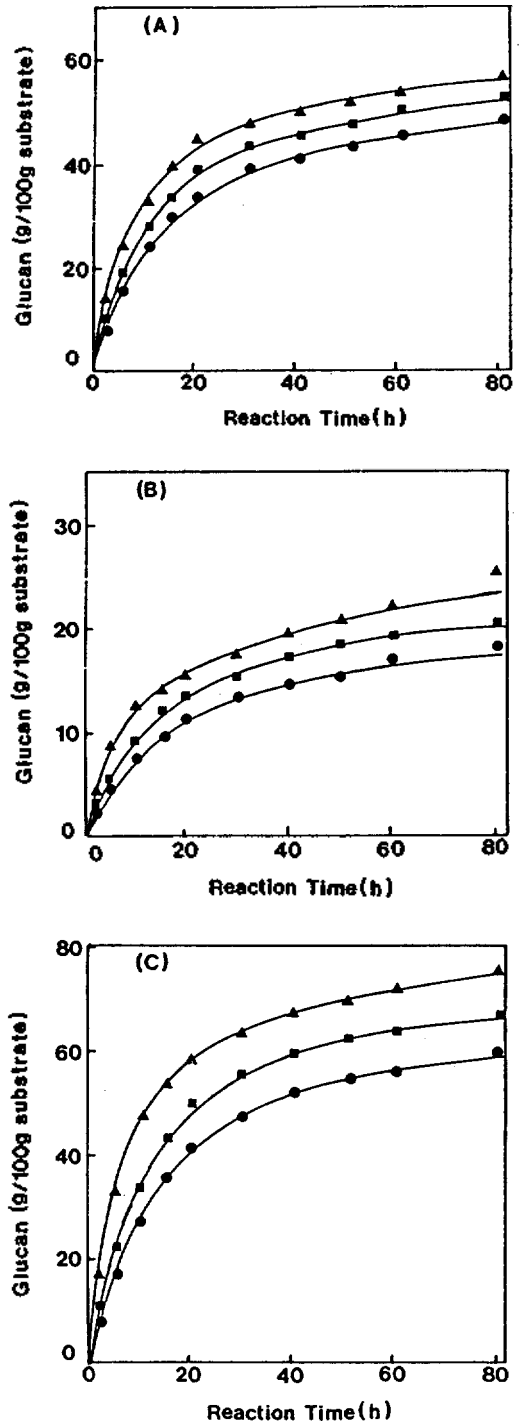

Figure 2. The relationship between the extent of hydrolysis of substate and reaction time. The solid lines are those calculated with the individual constants of Table 1, using Equation (4). The ordinate is 100 (Y/C_0). Substate; Sigmacell 100. Pretreatment in hr : 0; (▲), 12; (■), 48; (●). Enzyme : endoglucanase I; (A), exoglucanase II; (B), exoglucanase II in combination with endoglucanase I; (C).

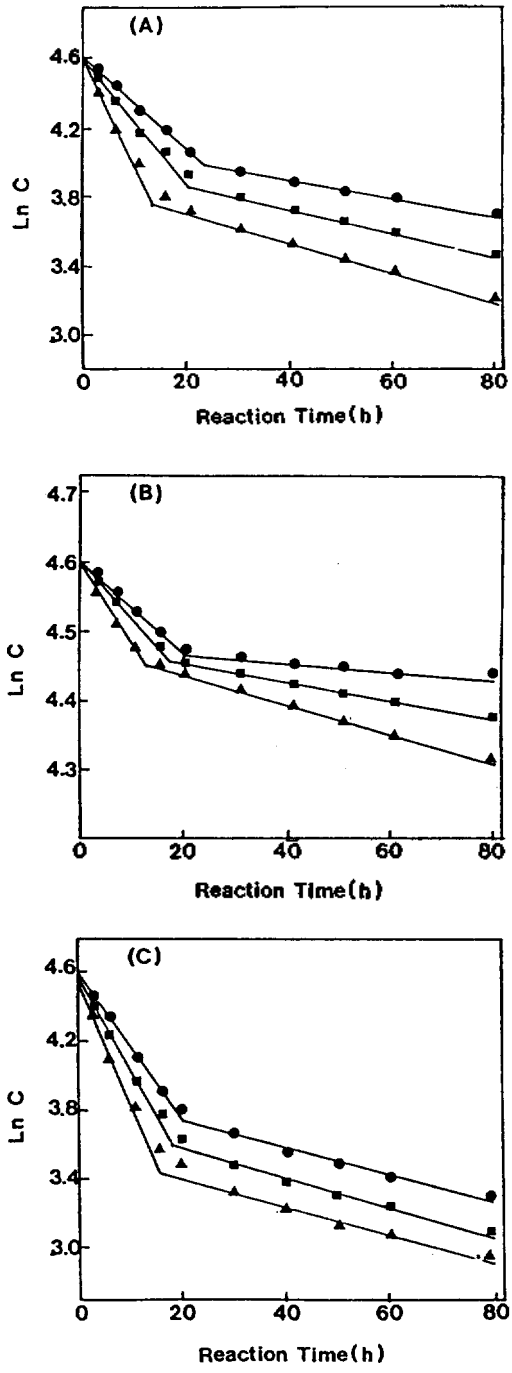

Figure 3. Semilogarithmic plots of residual unhydrolyzed substate versus reaction time. The ordinate is $\ln\{100(C_t/C_o)\}$. The symbols are the same as in Figure 2.

the substrate used for the enzymatic hydrolysis consists more or less entirely of cellulose. We have applied the nonlinear least square (NLS) methed,[30,31] using Equation (4) and the data set shown in Figure 2 to solve the Equation. This method allows to determine for each time curve in Figure 2, the rate constants k_a and k_b, and C_a/C_o and C_b/C_o. The solid lines in Figure 2 are those calculated by NLS method and it is evident that the experimental data are in a very good agreement with the calculated curve.

The percentages of easily (C_a) and difficult (C_b) hydrolyzable cellulose in Sigmacell 100 and associated rate constants k_a and k_b are given in Table 1. The value of the rate constant k_a is larger than that of rate constant k_b, therefore k_b has significant influence on the reaction rate for k_a. It is impor-

Table 1. Percentages of easily (C_a) and difficult (C_b) hydrolyzable Sigmacell 100 and associated rate constants, k_a and k_b. The values for 100 (C_a/C_o), 100 (C_b/C_o), k_a, and k_b were computed by nonlinear regression analysis for the time curves shown in Figure 2

Sigmacell 100		Endo I (A)		
Pretreatment Time (hr)	Percent C_a (%)	Percent C_b (%)	k_a $(h^{-1})\times10^0$	k_b $(h^{-1})\times10^2$
0	39	61	0.15	0.43
12	37	63	0.11	0.36
48	34	66	0.09	0.31
		Exo II (B)		
0	17	83	0.19	0.20
12	14	86	0.10	0.10
48	11	89	0.09	0.09
	Exo II in combination with Endo I (C)			
0	51	49	0.17	0.88
12	49	51	0.10	0.55
48	45	55	0.08	0.38

tant that the NLS gives for all curves measured nearly identical values for the fraction of easily and difficult hydrolyzable cellulose, as well as for the rate constant k_a. This means that C_a/C_o, C_b/C_o, and k_a are real constants, which are the same enzyme-substrate system independent from the different substrate. On the other hand, the rate constant k_b is dependent on the enzyme species and increases about 4 fold if the enzyme is used as, from Exo II to Exo II in combination with Endo I. The agreement between experimental values and those calculated using the respective constants in Table 1 can also be seen from the Figure 2. Therefore, we conclude that two parallel pseudo first order reactions are sufficient to describe the time dependency of the hydrolysis of cellulose at least in our study with Sigmacell 100 and Endo I, Exo II, and Exo II in combination with Endo I system.

From the Figure 2, we find that enzyme and substrate have a significant influence on the hydrolysis charateristics of celluloses. The hydrolysis curves for three different types of Sigmacell 100 showed charateristic differences when certain parameters such as the nature of the enzyme and enzyme composition are changed. A rapid increase in the amount of reducing sugar is observed during the hydrolysis period from 0 to about 12 hr. After about 12 hr, the hydrolysis rate is gradually increased than initial rate. For all cases, the amount of reducing sugar decreased with increasing the correlation crystallinity index. The surface area and the crystallinity of cellulose that affect the susceptibility of cellulose to enzymatic hydrolysis are very important.[20,53] The contact between the enzyme molecules and the surface of cellulose particles is a prerequisite for the hydrolysis to proceed, and that of the latter from the fact that the enzyme degrades the more accessible amorphous region of cellulose, more readily than the less accessible crystalline region. Therefore, as the crystallinity index increases, cellulose becomes increasingly resistant to further hydrolysis. On the other hand, the increased initial specific surface area enhances the extent of initial soluble protein adsorption, inceases the initial hydrolysis rate.[20,53,54]

The endoglucanase I and exoglucanase II have high affinity for the adsorption on microcrystalline cellulose.[55] But, the heat of adsorption of Endo I is much smaller than that of Exo II. This suggests that the endoglucancase does not affect adsorption of the exoglucanase significantly. It can be concluded that Exo II shows stronger preferential adsorption than Endo I.[18,55] The endoglucanase acts randomly, mainly on amorphous or modified cellulose such as carboxymethylcellulose and cello-oligomers, to give cellobiose as the major hydrolysis product. However, it is inactive on crystalline cellulose.[54,55] On the contrary, but, a more reactive endoglucanase produces more glucose. This is apparently formed as a result of transglucosylation catalyzed by the endoglucanase.[1] As can be seen in Figure 2(A), the extent of the hydrolysis is relatively larger, compared with the exoglucanase II shown in Figure 2(B). The exoglucanase removes a cellobiose unit from the nonreducing end of the cellulose chain. This component attacks neither the amorphous or modified cellulose nor the crystalline cellulose to any significant extent.[54,55] Therefore, the relatively small amount of reducing sugar is due to the naturally being nonreducing end of the cellulose chain.[44] However, once this component is combined with the endoglucanase component and β-glucosidase, it plays a major role in the hydrolysis of highly crystalline cellulose.[54,56] It was found that Exo II in combination with Endo I had a synergistic effect in the hydrolysis of microcrystalline cellulose.[56] This indicates that the portion of Endo I in the enzyme mixture may provide a sufficient number of chain ends for an Exo II to act on.[55] Figure 2(C) shows that the largest amount of reducing sugar for the hydrolysis of Sigmacell 100 is due to the synergism of Exo II in combination with Endo I.

As shown in Table 1, the C_a values decreased with increase in the relative crystallinity index of the substrate, while the C_b values is increased. These results suggest that the number of reaction sites for each emzyme decreases with increasing the crystallinity index of substrate. In the case of Endo I, the k_a values increased with increasing the C_a values, while the k_b values decreased with increasing the C_b values. The k_a values are about 10^2 fold greater than those of the k_b, in general. This result indicates that the reaction of the difficult hydrolyzable portion of cellulose is the rate determining step. On the other hand, the k_a and k_b values are decreased with an increase in the crystallinity in all enzyme-substrate systems. However, in Exo II-substrate system, the C_b values are very large, as compared with another systems. This means that the enzyme action of Exo II towards cellulose is not effective by alone. A drastic increase of the k_b values in Exo II in combination with Endo I system is due to the synergistic effect related enzymes.[1,54-56] Crystalline cellulose is degraded by a synergistic action of endoglucanases creating nicks in the anhydroglucose chains and free chain ends are created, and exoglucanases removing cellobiose from the nonreducing ends of the cellulose chains.[57,58]

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