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Saccharification of Cellulose by Combined Hydrolysis with Acid and Enzyme*

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Acid hydrolysis of cellulose in heterogeneous and homogeneous systems was studied theoretically and experimentally. In the heterogeneous system, solubilization of cellulose was the rate-limiting step. The low yield of glucose was due to decomposition of glucose and cellooligosaccharides produced from cellulose to other materials than sugars during the long exposure to strong acid. In the homogeneous system, cellulose was hydrolyzed at random, and the rate constant of the hydrolysis k_c(hr^{-1}) at 50°C was expressed by the following equation.

$$k_{c,50°C} \fallingdotseq 9.4 \times 10^{-8} \exp (0.28\ [HCl])$$

where [HCl] represents the concentration of hydrochloric acid in the range of 4 to 16(w/w)%. The activation energy for the hydrolysis of cellulose was 27.8 kcal/mol.

The thermal stability of cellulase immobilized on Duolite ES-762 exceeded that of free cellulase. The pH optima of immobilized and free cellulases were in the range of 4.0 to 5.0. The Michaelis constant K_m of immobilized cellulase approximated to that of free cellulase.

The total amount of cellulose used was converted first into glucose and soluble cellooligosaccharides by acid hydrolysis in the homogeneous system, and then completely into glucose by hydrolysis with the immobilized cellulase.

Many methods have been proposed for hydrolysis of cellulose to utilize it as an energy source for production of single-cell protein and other useful substances.[1,2] It is, however, very difficult to hydrolyze native cellulose effectively with cellulase, since cellulose is insoluble in water. To obtain a high yield of glucose at a high reaction rate, high concentrations of cellulase and physical or chemical pretreatment of cellulose are required.[3~7] However, because of the substrate specificity of enzymatic hydrolysis, undesirable by-products are not produced.

In acid hydrolysis in a heterogeneous system, solubilization of cellulose appears to be the rate-limiting step, and to accelerate this step, high concentrations of strong acid[8~10] and high temperatures[11~14] are necessary. Moreover, glucose produced early in the reaction is exposed to strong acid for a long time and decomposes to levulinic acid, formic acid etc., thus depressing the yield of glucose. To overcome these demerits, hydrolysis should be performed in a homogeneous system in which cellulose is dissolved completely.

Cellulose is soluble in highly concentrated solutions of phosphoric acid, cadoxen, zinc chloride etc.[8] But hydrolysis of cellulose in these solutions is very slow. However, by adding strong acid, such as hydrochloric acid, to the cellulose solution, the hydrolysis of cellulose is markedly accelerated. Although both dissolution and hydrolysis of cellulose can be achieved in highly concentrated sulfuric acid, use of the acid is best avoided, since the excessively high rate of hydrolysis and high heat of dilution raise operational

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problems, for example, in controlling the final degree of reaction to produce the desired yield of soluble cellooligosaccharides. In the homogeneous system, cellulose is hydrolyzed at random, as in the case of starch hydrolysis, and is converted very rapidly into cellooligosaccharides with an average degree of polymerization at which they are soluble in water. Moreover, the rate of hydrolysis in the homogeneous system is higher than that in the heterogeneous one at the same concentration of strong acid and temperature. Although it is disadvantageous to use high concentrations of phosphoric and hydrochloric acids, the former is necessary for the homogeneous reaction and the latter is necessary to increase the rate of hydrolysis. By use of these acids, a high yield of soluble cellooligosaccharides can be obtained at a high reaction rate without further decomposition of the product.

Considering the high rate of acid hydrolysis in the homogeneous system and high substrate specificity of enzymatic hydrolysis, we devised a combined hydrolysis system with acid and enzyme to saccharify cellulose to glucose in high yield. This system consists of three steps: 1) dissolution of cellulose in phosphoric acid solution, 2) homogeneous acid hydrolysis of dissolved cellulose with hydrochloric acid to soluble cellooligosaccharides, and 3) hydrolysis to glucose by immobilized cellulase.

To determine the feasibility of the system, we have investigated the kinetics of homogeneous acid hydrolysis in comparison with the heterogeneous hydrolysis and examined the properties of immobilized cellulase. Economic problems such as recovery and recycling of phosphoric and hydrochloric acids will be examined in future work.

Materials and Methods

Materials Crystalline cellulose (powder, No. 2330) was purchased from E. Merck AG., glucostat reagent from Worthington Biochemical Corp., and cellobiose from Wako Pure Chemical Industries, Ltd. Duolite ES-762 (porous phenolic resin) from Diamond Shemrock Co. was generously given by Sumitomo Chemical Co. Ltd. A crude cellulase preparation was given by the Central Research Institute of Ishihara Sangyo, Co. Ltd., which had been prepared from the culture filtrate of *Pellicularia filamentosa* strain FERM-P1797 by precipitation with ammonium sulfate.[8]

Immobilization of cellulase Cellulase was immobilized by essentially the same method as that for immobilization of lactase.[15] One g of Duolite ES-762 (wet) equilibrated with 0.2 M acetate buffer (pH 5.0) at 5°C for 24 hr was suspended in 100 ml of the same buffer containing the crude cellulase in an initial concentration range of from 1.5 to 9 mg/ml. The suspension was shaken at 30°C for 48 hr, then the resin was washed well with the same buffer. Two hundred mg of resin with adsorbed cellulase was resuspended in 10 ml of the same buffer containing glutaraldehyde in a final concentration range of from 0.5 to 10 (w/w)% and shaken at 5°C.

Hydrolysis of cellulose For acid hydrolysis in the heterogeneous system, 20 ml of 24 mg/ml crystalline cellulose suspension in 28.1 (w/w)% hydrochloric acid was hydrolyzed at 60°C with stirring. The hydrolysate was centrifuged at 2,000×*g* for 10 min, and the supernatant was diluted with distilled water in an ice-water bath to stop the hydrolysis.

For acid hydrolysis in the homogeneous system, 0.30, 0.24, 0.18, 0.16 or 0.15 g of crystalline cellulose was suspended homogeneously in 0.5 ml of distilled water. The cellulose was dissolved by adding 8 ml of 85.0 (w/w)% phosphoric acid, then hydrolyzed with 10, 6.3, 2.6, 1.37 or 0.75 ml of 35.0 (w/w)% hydrochloric acid. The reaction was allowed to proceed in the temperature range of from 30 to 55°C for an appropriate period. The hydrolysate was adjusted to approximately pH 5 with 4 N sodium hydroxide to stop the hydrolysis, then centrifuged at 2,000×*g* for 10 min. The supernatant was used for the enzymatic hydrolysis without further treatment. One hundred mg of Duolite ES-762 (wet) on which cellulase was immobilized was used per ml of the supernatant for the enzymatic hydrolysis. The reaction was allowed to proceed at 40°C.

Decomposition of glucose Twenty ml of 6.4 mg/ml glucose dissolved in 28.1 (w/w)% hydrochloric acid was decomposed at 60°C for an appropriate period. The hydrolysate was diluted with distilled water in an ice-water bath to stop the reaction.

Analytical methods Total sugar and glucose were determined by the phenol-sulfuric acid method[16] and the glucostat method with glucose as standard, respectively. Protein was estimated by Lowry's method[17] with crystalline bovine serum albumin as standard. The degree of solubilization of cellulose was defined as the ratio of the amount of total sugar produced in the supernatant after the stopping of hydrolysis to the initial amount of cellulose. The

activity of free cellulase was assayed in the reaction mixture containing 2 ml of 11 mg/ml cellobiose in 0.2 M acetate buffer (pH 5.0) and 0.2 ml of the cellulase, and the reaction was stopped by adding 0.25 N sodium hydroxide. The activity of immobilized cellulase was assayed in the reaction mixture containing 2.2 ml of 10 mg/ml cellobiose in the same buffer and 20 mg of Duolite ES-762 (wet) with shaking. To obtain the Michaelis constant K_m, a cellobiose solution of an appropriate concentration was prepared with 0.2 M acetate buffer (pH 5.0). The reaction was allowed to proceed at 30°C. The reaction mixture consisted of 5 ml of the substrate solution and 0.5 ml of the crude cellulase solution adjusted to 6 mg protein/ml with the same buffer in the case of free cellulase, and 8 ml of the substrate solution and 20 mg of Duolite ES-762 (wet) on which 0.27 mg protein was immobilized per mg of the resin in the case of immobilized cellulase. The reaction was stopped by adding 0.25 N sodium hydroxide. One unit of cellulase activity is defined as the amount of the enzyme producing 1 μmole of glucose from the substrate per min at 30°C.

Results and Discussion

Acid hydrolysis in the heterogeneous system Twenty ml of 24 mg/ml crystalline cellulose suspension in 28.1 (w/w)% hydrochloric acid was hydrolyzed at 60°C. The result is shown in Fig. 1. Since the difference between the amounts of total sugar and glucose was relatively small, the solubilization of cellulose seemed to be a rate-limiting step. In the early stage the rate of hydrolysis was

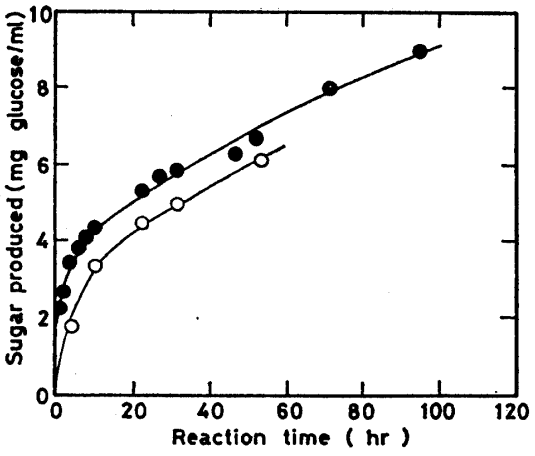

Fig. 1. Time course of acid hydrolysis of cellulose in the heterogeneous system.
Twenty ml of 24 mg/ml crystalline cellulose suspension in 28.1 (w/w)% hydrochloric acid was hydrolyzed at 60°C for the indicated period with stirring.
Symbols: ●, total sugar; ○, glucose.

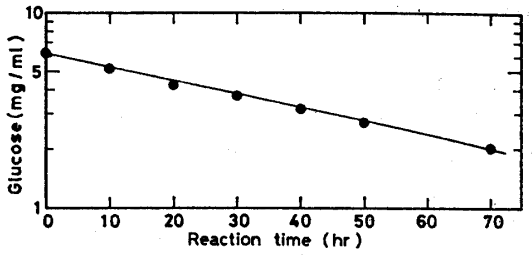

Fig. 2. The rate of decomposition of glucose.
Twenty ml of 6.4 mg/ml glucose dissolved in 28.1 (w/w)% hydrochloric acid was decomposed at 60°C for the indicated period.

much faster than that in the late stage. This indicates that cellulose has amorphous and crystalline regions, which show different reactivities in acid hydrolysis. Figure 1 also shows that total sugar solubilized increases almost linearly in both stages. This indicates that the reaction can be assumed to be of zero-order except at high conversion. Figure 2 shows the rate of decomposition of 6.4 mg/ml glucose under the same conditions. The decomposition of glucose to other materials than sugars was a first-order reaction with a rate constant of 1.6×10^{-2} (hr^{-1}).

From these results, the yield of glucose containing a small amount of cellooligosaccharides can be estimated by the following procedure. The following assumption are made: 1) The solubilization of cellulose is a rate-limiting step in the heterogeneous reaction system, and the cellulose solubilized is converted into glucose and a small amount of cellooligosaccharides with a rapid velocity. 2) Cellulose consists of amorphous and crystalline regions. The solubilization of cellulose in both regions is a zero-order reaction. 3) The decomposition of glucose and cellooligosaccharides to other materials than sugars is a first-order reaction with the rate constant obtained from Fig. 2, because the amount of cellooligosaccharides produced is very small in comparison with that of glucose. From these assumptions, the following equations can be derived.

$$-dC_a/d\theta = k_a \quad (1)$$

$$-dC_r/d\theta = k_r \quad (2)$$

$$dS_m/d\theta = (k_a+k_r) - k_mS_m \quad (3)$$

$$(C_t)_0 = (C_a)_0 + (C_r)_0 \quad (4)$$

where $(C_a)_0$ and $(C_r)_0$ (g/ml) are the concentrations of cellulose in amorphous and crystalline regions at the beginning of reaction, respectively, and $(C_t)_0$ (g/ml) is the initial concentration of cellulose. C_t (g/ml) is the concentration of cellulose at an arbitrary reaction time θ(hr). C_a and C_r (g/ml) are the concentrations of cellulose in amorphous and crystalline regions, respectively, and S_m (g/ml) is the concentration of glucose and cellooligosaccharides at an arbitrary reaction time. k_a and k_r (g/ml/hr) represent the rate constants of solubilization of cellulose in amorphous and crystalline regions, respectively, and k_m (hr^{-1}) is the rate constant of decomposition of glucose and cellooligosaccharides as described above. The concentrations $(C_a)_0$ and $(C_r)_0$ and the coefficients other than k_m were determined by trial-and-error to fit the concentrations of total sugar calculated from the measured values shown in Fig. 1. These coefficients and concentrations determined were $k_a=3.8\times10^{-3}$, $k_r=1.7\times10^{-4}$, $(C_a)_0=2.7\times10^{-3}$ and $(C_r)_0=2.1\times10^{-2}$. The yield of glucose with a small amount of celloligosaccharides calculated from these coefficients and concentrations $(C_a)_0$ and $(C_r)_0$ was approximately 40% when the degree of solubilization of cellulose was 100%. This indicates that acid hydrolysis of cellulose in the heterogeneous system causes a significant loss of glucose.

Acid hydrolysis in the homogeneous system By assuming that cellulose dissolved homogeneously in phosphoric acid is hydrolyzed at random with hydrochloric acid, and that the first-order rate constant of bond cleavage in acid hydrolysis k_c (hr^{-1}) is independent of the degree of polymerization (DP) of cellulose $n(-)$, the rate of increase of the concentration of cellulose C_n (mole/ml) with a DP of n is expressed by Eq. (5).

$$dC_n/d\theta = -k_c(n-1)C_n + 2k_c\sum_{x=n+1}^{N} C_x \quad (5)$$

where C_x (mole/ml) represents the concentration of cellulose with DP of $x\geqq n+1$, N the maximum DP of cellulose used, and θ(hr) reaction time. The first term in the right hand side represents the rate of decrease of the concentration of cellulose with a DP of n, and the second term the rate of increase of the concentration of cellulose with a DP of n formed from cellulose with a DP equal to or greater than $n+1$. If cellulose with a DP equal to or less than N-1 is absent at the beginning of the reaction, the ratio of the concentration of cellulose with a DP of N at an arbitrary reaction time C_N (mole/ml) to the initial concentration of cellulose C_{N_0}(mole/ml) is expressed by Eq. (6), and for cellulose with an arbitrary DP of $i(-)$, Eq. (7) can be derived from Eqs. (5) and (6).

$$C_N/C_{N_0} = \exp(-k_c(N-1)\theta) \quad (6)$$

$$C_i/C_{N_0} = (N+1-i)\exp(-(i-1)k_c\theta) - 2(N-i)\exp(-ik_c\theta) + (N-1-i)\exp(-(i+1)k_c\theta) \quad (7)$$

where i in Eq. (7) is defined as a positive integer from 1 to $N-1$. If the maximum DP of cellulose is sufficiently high, and celluloses (in practice, cellooligosaccharides) with DP equal to or less than l are soluble after the stopping of hydrolysis, the ratios of the weights of glucose $G_g(-)$ and total sugar $S_t(-)$ in the supernatant to the initial weight of cellulose can respectively be expressed approximately by Eq. (8), by substituting 1 for i in Eq. (7), and by Eq. (9), by summing up iC_i for i values from 1 to l.

$$G_g = C_1/N\cdot C_{N_0} \fallingdotseq (1-\exp(-k_c\theta))^2 \quad (8)$$

$$S_t = \sum_{i=1}^{l} iC_i/N\cdot C_{N_0} \fallingdotseq 1 - (l+1)\exp(-lk_c\theta) + l\exp(-(l+1)k_c\theta) \quad (9)$$

These quantities (G_g and S_t) can also be obtained by experiment.

Figure 3 shows typical time courses of acid hydrolysis of cellulose in a homogeneous system. It is clear by comparing Fig. 3 with Fig. 1 that the reaction rate of hydrolysis in the homogeneous system was much higher than that in the heterogeneous system. In the homogeneous system, cellulose was completely solubilized much more quickly, and thus the degree of decomposition of glucose may be greatly reduced. The rate con-

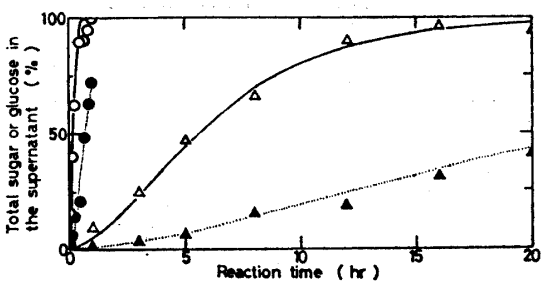

Fig. 3. Time course of acid hydrolysis of cellulose in the homogeneous system.
Crystalline cellulose (0.30 g) was suspended homogeneously in 0.5 ml of distilled water. The cellulose was dissolved in 8 ml of 85.0 (w/w)% phosphoric acid, and hydrolyzed with 10 ml of 35.0 (w/w)% hydrochloric acid. The reaction was allowed to proceed at 30°C or 55°C. The ordinate indicates the ratio of the amount of glucose (▲, 30°C; ●, 55°C) or total sugar (△, 30°C; ○, 55°C) in the supernatant after the stopping of the hydrolysis to the initial amount of cellulose.

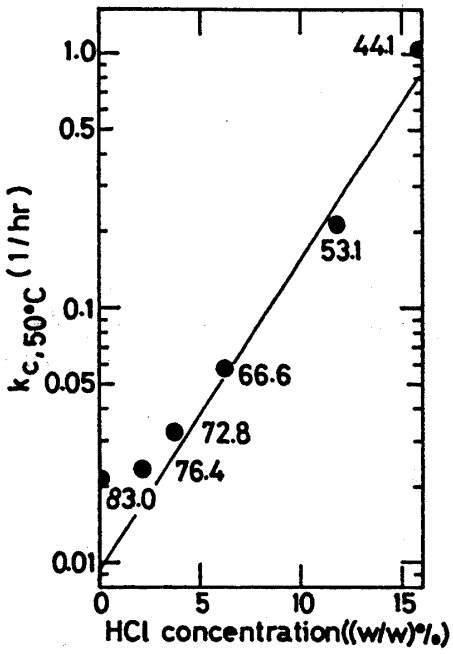

Fig. 4. Effect of concentration of hydrochloric acid on the rate constant of acid hydrolysis of cellulose.
The concentration of crystalline cellulose was constant at about 1 (w/w)%. The reaction temperature was 50°C. The values in this figure are the concentrations of phosphoric acid (w/w)%.

stants of the hydrolysis with 16 (w/w)% hydrochloric acid and 44 (w/w)% phosphoric acid at various temperatures were determined by substituting the amounts of glucose measured at arbitrary times for G_t in Eq. (8). The values obtained were $k_{c,30°C}=5.4\times10^{-3}$, $k_{c,35°C}=1.1\times10^{-1}$, $k_{c,40°C}=2.4\times10^{-1}$, $k_{c,45°C}=4.6\times10^{-1}$, $k_{c,50°C}=1.0$ and $k_{c,55°C}=1.8$. As shown in Fig. 3, the G_t values (dotted curves) obtained from Eq. (8) by using these k_c values were in good agreement with those measured. An Arrhenius plot of these values was linear, and the activation energy for the hydrolysis of cellulose was determined to be 27.8 kcal/mol. Figure 4 shows the effect of concentration of hydrochloric acid on the rate constant of acid hydrolysis of cellulose at 50°C. Since the rate constant of the hydrolysis only with 83.0 (w/w)% phosphoric acid was relatively small, 0.0215 (hr^{-1}), k_c could be approximately expressed as a function of the concentration of hydrochloric acid as follows.

$$k_{c,50°C} \fallingdotseq 9.4\times10^{-3}\exp(0.28[HCl]) \quad (10)$$

where [HCl] represents the concentration of hydrochloric acid, which is in the range of 4 to 16 (w/w)%. For very dilute solutions of hydrochloric acid, the value calculated from Eq. (10) did not agree with the measured value, because the hydrolysis with phosphoric acid became a dominant factor. Cellooligosaccharides of DP less than 7 are reportedly water-soluble.[18] However, the best fit with measured values of S_t curves calculated from the rate constants of acid hydrolysis given above (solid curves in Fig. 3) was obtained by assuming $l=5$ in Eq. (9). Although it is not clear why l should be 5 rather than 7, at present, it may be ascribed to the high concentration of the salt solution after the reaction is stopped. This suggests that the assumptions that the hydrolysis proceeds at random and that k_c is not influenced by the DP of cellulose are reasonable. Incidentally, crystalline cellulose only dissolved in 83.0 (w/w)% phosphoric acid was solubilized up to 20% at 30°C over 20 hr (data not shown), and crystalline cellulose hydrolyzed with hydrochloric acid after dissolution in phosphoric acid almost completely hydrolyzed under the conditions given in Fig. 3 within 20 hr and 35 min respectively at 30°C and 55°C. The number average degree of polymerization $\overline{DP}_n$ is expressed as

follows by use of Eqs. (6) and (7).

$$\overline{DP}_n = \sum_{i=1}^{N} iC_i / \sum_{i=1}^{N} C_i$$
$$= 1/(1-(1-1/N)\exp(-k_c\theta)) \quad (11)$$

The value of $\overline{DP}_n$ calculated from Eq. (11) for almost completely solubilized cellulose was approximately 1.5.

Properties of immobilized cellulase Since crystalline cellulose was completely converted into soluble cellooligosaccharides by acid hydrolysis in the homogeneous system, the use of an immobilized cellulase seemed to offer an effective means to further convert it to glucose. Therefore, the properties of immobilized cellulase were compared with those of the free enzyme. Cellobiose was used as the substrate for measurement of cellulase activity, since the $\overline{DP}_n$ value of the solution which would be subjected to subsequent enzymatic hydrolysis was low, as described above.

The activity of cellulase adsorbed on Duolite ES-762 was 5.5 units/g resin (wet), when the concentration of protein in the solution under adsorption equilibrium was in the range of 1 to 6 mg/ml. This value was much lower than that calculated from the activity of free cellulase remaining in the solution, 20—25 units/g resin (wet) (data not shown). There is no simple explanation for this difference at present. The effect of the concentration of glutaraldehyde on immobilization was investigated over 5 hr of reaction with shaking at 30°C. The activity of immobilized cellulase was scarcely decreased at concentrations of glutaraldehyde less than 2 (w/w)%, that is, 5.5 units/g resin (wet) (data not shown). The activity of cellulase immobilized at a glutaraldehyde concentration of 2 (w/w)% at 30°C was 90% of the original activity, that is, 5.0 units/g resin (wet), at 10 hr of reaction (data not shown).

Figure 5 shows that the pH optima for the immobilized and free cellulases are in the range of 4.0 to 5.0. The immobilized cellulase showed greater activity than the free enzyme at higher pH and similar activity at lower pH.

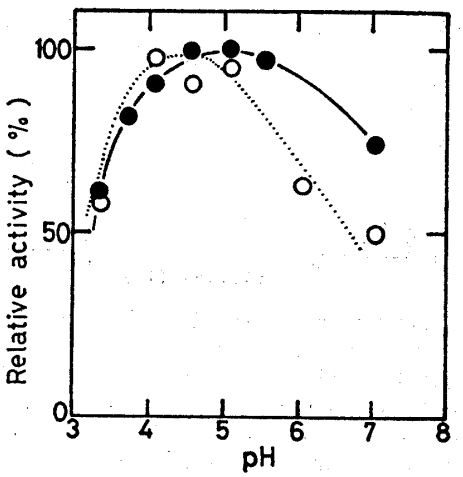

Fig. 5. Effect of pH on cellulase activity.
The immobilized and free cellulases were adjusted to appropriate pH values with 0.04 M Britton-Robinson's wide range buffer, and allowed to react at 30°C for 30 min. Cellulase activitty was determined according to "*Analytical methods*" except for the use of the buffer described above. Symbols: ○, free cellulase; ●, immobilized cellulase.

The immobilized and free cellulases were stable over the range of pH 3.0 to 7.0 when incubated for 150 hr at 30°C, and the former in particular was hardly inactivated (data not shown).

The thermal stability of the immobilized and free cellulases was investigated at 65°C. The results are shown in Fig. 6. The activities of the immobilized and free cellulases incubated for 2.5 hr were 25 and 5% of the respective original activities. The in-

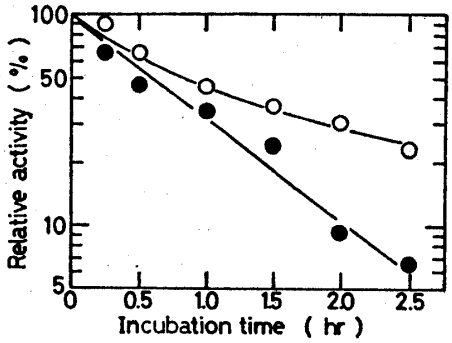

Fig. 6. Thermal stability of free and immobilized cellulases.
Enzymes were incubated at 65°C for the designated period in 0.2 M acetate buffer (pH 5.0), then assayed for retention of activity. Cellulase activity was determined according to "*Analytical methods.*" Symbols: ●, free cellulase; ○, immobilized cellulase.

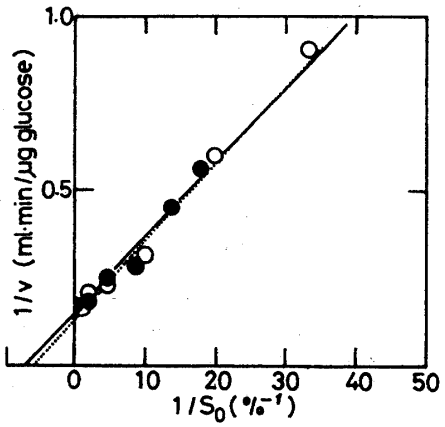

Fig. 7. Lineweaver-Burk plots for free and immobilized cellulases.
Reaction conditions were given in "*Analytical methods.*" S_0 (w/w)% and v (μg glucose/ml·min) denote the initial concentration of substrate and the initial velocity, respectively. Symbols: ○, free cellulase; ●, immobilized cellulase.

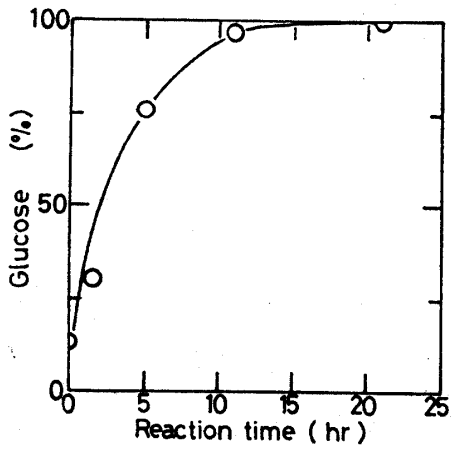

Fig. 8. Enzymatic hydrolysis of soluble cellooligosaccharides obtained by acid hydrolysis of cellulose in the homogeneous system.
The reaction temperature was 40°C. The reaction mixture was composed of 5 ml of the soluble cellooligosaccharides (total sugar, 3.2 mg/ml; glucose, 0.4 mg/ml) and 0.5 g of the immobilized cellulase (0.4 units/ml of the reaction mixture).

activation of free cellulase was a first-order reaction, but that of immobilized cellulase was not. A possible explanation for the non-first-order inactivation might be the heterogeneity of immobilized cellulase caused by the difference in the number of cross-links between cellulase and resin and between cellulases.[19]

The Michaelis constant K_m of the immobilized and free cellulases was 0.18 and 0.15 (w/w)% cellobiose, respectively, as given in Fig. 7. Obviously, the K_m value of the free cellulase was nearly equal to that of the immobilized one. This indicates that the hydrolysis with immobilized cellulase is not influenced by the mass-transfer rates of the substrate and products.

Enzymatic hydrolysis Soluble cellooligosaccharides obtained by acid hydrolysis in the homogeneous system (total sugar, 3.2 mg/ml; glucose, 0.4 mg/ml) were hydrolyzed at 40°C with the immobilized cellulase (0.4 units/ml of the reaction mixture). Figure 8 shows that the cellooligosaccharides were completely converted into glucose within 10 hr of reaction. This indicates that the decomposition of glucose and cellooligosaccharides produced by the acid hydrolysis is negligible during the solubilization of crystalline cellulose to soluble cellooligosaccharides.

Crystalline cellulose is completely converted into glucose by the suitable combination of acid hydrolysis in the homogeneous system with enzymatic hydrolysis with immobilized cellulase. Further, the continuous production of glucose from cellulose is possible by use of immobilized cellulase.

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