Kinetic Study on the Production of Cyclodextrin: Analysis of Initial Rate, Inhibitory Effect of Glucose and Degradation

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Abstract—CGTase (Cyclodextrin Glycosyltransferase) was used as an enzyme to produce CD (Cyclodextrin). Reducing sugar was one of the main by-products during the reaction and showed an inhibitory effect on the formation of CD. In the experiment, glucose was used to replace reducing sugar. When the concentration of glucose is very low, its effect can be neglected and a Michaelis-Menten model used to describe the reaction. The parameters V_{max} (maximum reaction rate) and K_m (Michaelis-Menten constant) were obtained by a Lineweaver-Burke plot. When the concentration of equivalent glucose increases, its inhibitory effect can be observed. The "mixed-type inhibitory reaction" illustrated that the inhibitory effect was caused by high glucose concentrations. The reaction parameters can be found again by the graphic method. Glucose not only prevented the formation of CD but also combined with CGTase to destroy the produced CD. This degradation reaction is one kind of enzymatic reaction. The kinetics of the reaction leading to the production of CD will be investigated.

INTRODUCTION

Cyclodextrin (CD) is a non-reducible polysaccharide. Each polysaccharide is composed of 6 to 12 glucose molecules with alpha-1, 4 glucoside linkage. There are three major kind of CD, *i.e.*, α-, β-, and γ-CD which contain 6, 7, and 8 glucose molecules respectively. Since the solubility of β-CD is much lower than that of α- and γ-CD, it is more easily separated than the others. The structure of CD looks like a hollow cylinder. The host molecule has the property of receiving the guest molecule into its cavity and becomes an inclusive compound. CD can modify the physical or chemical properties of the guest molecule and serve as a stabilizer. CD also can be used as additive in medicine, cosmetics, anticide and food industry[3,8,9,12,13].

In 1976, Nakamura and Horikosei[8] selected the extracellular CGTase-producing strain Alkalophilic *Bacillus sp.*, ATCC 21783. They found that the CGTase produced by alkalophilic *Bacillus* had the advantage of producing less undesired oligosaccharides with low molecular weights, thus the yield of CD was greatly promoted[5~7]. The CGTase produced by *Bacillus sp.* No. 38-2 (ATCC 21783) had good stability at pH 6-9 and 50-60°C; therefore, it could be applied to industrial production[3,9,10]. Industrial procedures in CD production were developed by Horikoshi *et al.*[1,2,4].

Recently, M. Nonoto *et al.*[11] had succeeded in isolating a strain of alkalophilic *Bacillus sp.* No. HA 3-3-2 from some soil in Taipei, which could produce a sufficient amount of CGTase in the culture at high pH values. The properties of CD and its applications have been investigated[8,10]; however, none of the reaction kinetics that is important for the reactor design is mentioned. This work studied a kinetic model for the CD formation.

EXPERIMENTAL

1. Materials

Potato starch was purchased from Denmark,

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CGTase was purchased from Meito Sangyo Co., Japan (activity=33,000 U/g). CGTase solution was prepared by mixing 1 g of CGTase power in 80 ml of distilled water and the supernatent collected after centrifuge. Then the solution was stored at 4°C. α-amylase was purchased from Sigma Co., U. S. A. (activity=73,000 U/g). The α-amylase solution was prepared by mixing 1 g of α-amylase powder with 400 ml of distilled water. The supernatent was collected, then stored at 4°C. The DNS solution was prepared by mixing 0.5 g of DNS (Fluka Co.), 20 ml of 2.0 N NaOH solution, and 30 g of Rochelle salt with 100 ml of distilled water.

2. Experimental procedures

(1) Preparation of soluble starch

500 ml of 5% (w/v) potato starch, 0.55 g $CaCl_2$ and 6.6 ml of CGTase solution were mixed and kept at 85°C for 40 min and 100°C for 10 min successively to inactivate the enzyme, then the soluble starch was obtained.

(2) Formation of CD

The pH of the soluable startch solution was adjusted to 8.5. The CGTase solution was added (2.625×10^{-4} g/ml), and the mixture was kept at 55°C. 15 ml samples were taken for analysis at proper time intervals.

(3) Stop reaction and analysis of CD

The samples were boiled for 5 min to deactivate the CGTase, then 45 μl of α-amylase solution was added to hydrolyze the unreacted starch at 95°C for 10 min. T. C. E. (trichloroethylene) method was applied to analyze CD quantitatively as follows: the sample solution (15 ml) was cooled to room temperature, then completely mixed with 9 ml of T. C. E.. CD would be fully precipitated after keeping the mixture overnight. The precipitate was collected by filtration, dried in vacuum dryer and weighed. DNS method was used to analyze the reducing sugar. The procedures were repeated under various reaction conditions.

(4) Inhibitory effects of glucose

Various amounts of glucose were added as the equivalent reducing sugar at the beginning of reaction. The procedure was the same as above (cnto (3)).

(5) DNS method

5 ml of sample was mixed with 5 ml of DNS solution and kept in boiled water for 10 min. The mixture was then analyzed by UV spectroscopy, using optical density of 500 nm and glucose as the standard.

RESULTS AND DISCUSSIONS

1. The formation rate of CD without considering glucose effect

Figure 1 shows the relation of the CD formation concentration and time for various starch concentrations at 55°C. It was found that the higher concentration of starch, the longer the time needed to reach equilibrium and the larger amount of CD produced. The trace amount of CD obtained from the starch liquefying process was deduced. Figure 2 shows the relation between starch concentrations and CD initial formation rates. When the inhibitory

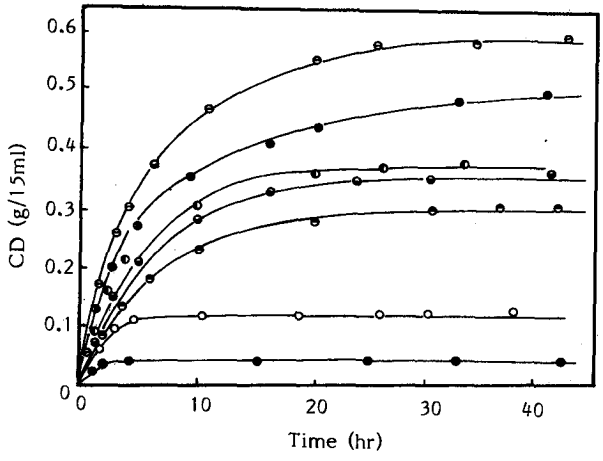

Fig. 1. Formation of CD at various concentrations of starch at 55°C. Starch concentration (g/l)= 10 (●), 25 (○), 50 (◓), 75 (◒), 100 (◐), 150 (◑), 200 (⊖).

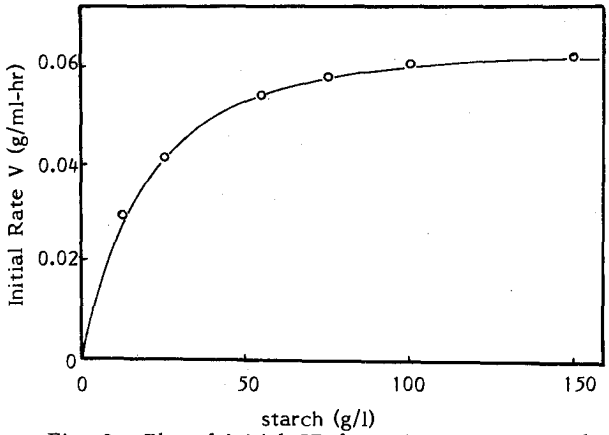

Fig. 2. Plot of initial CD formation rate *vs.* starch concentration.

effect was neglected at the beginning of the reaction, the kinetics of CD formation reaction could be expressed as follows:

$$E+S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} ES \xrightarrow{K_p} E+P \qquad (1)$$

where E: CGTase, S: starch, ES: intermediate, P: cyclodextrin, K_{+1}, K_{-1}, K_p: rate constants.

The rate equation could be expressed as Eq. (2)

$$V=\frac{V_{max}\times[S]}{K_m+[S]} \qquad (2)$$

where K_m is the Michaelis-Menten constant $(=(K_{-1}+K_p)/K_{+1})$, V_{max} is the Maximum reaction rate $(K_p[E_0])$, and $[S]$ is the starch concentration (g/ml).

Equation (2) also could be rewritten as Eq. (3)

$$\frac{1}{V}=\frac{K_m}{V_{max}}\times\frac{1}{[S]}+\frac{1}{V_{max}} \qquad (3)$$

Based on Fig. 2, the Lineweaver-Burke plot ($1/v$ *vs.* $1/s$) was shown in Fig. 3, in which the slope $(=K_m/V_{max})$ was 0.228 and the intercept $(=1/V_{max})$ was 14.0; therefore, $V_{max}=0.07118(g/ml\text{-}hr)$, $K_m=0.01623$ (g/ml) and the rate equation of CD formation reaction could be expressed as follows:

$$V=\frac{0.07118\times[S]}{0.01623+[S]} \qquad (4)$$

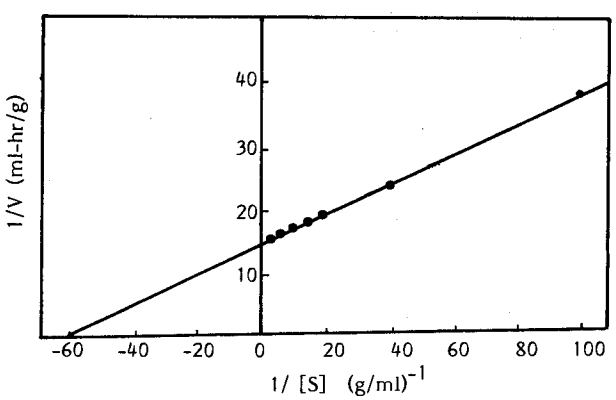

Fig. 3. Lineweaver-Burk plot of Fig. 2.

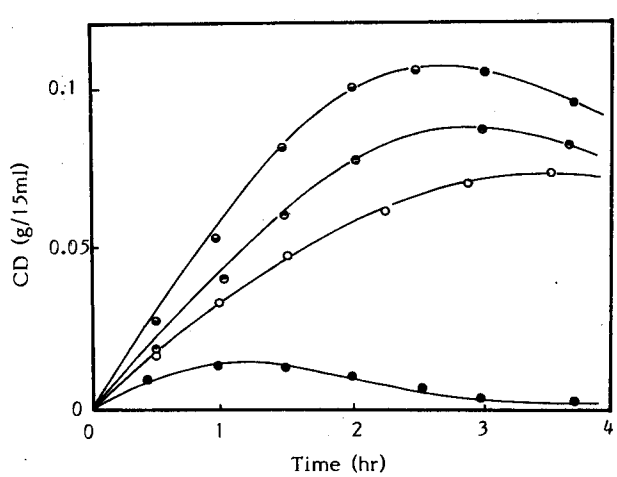

Fig. 4. Formation of CD at different starch concentration in the presence of 0.01 g/ml glucose. Starch concentration wt%=2 (●), 5 (○), 10 (◓), 15(◒).

2. The formation rate of CD with glucose inhibitory effect

The glucose inhibitory effect of CGTase-catalysed was assumed to have the following mechanism:

$$\begin{array}{ccc} E+S & \overset{K_s}{\rightleftharpoons} & ES \overset{K_p}{\rightleftharpoons} E+P \\ + & & + \\ I & & I \\ \Big\Updownarrow K_i & a\times K_s & \Big\Updownarrow a\times K_i \\ EI+S & \rightleftharpoons & ESI \end{array}$$

where E: CGTase, I: glucose, P: cyclodextrin, ES, EI, and ESI: Intermediates, a: constant.

Figures 4 and 5 illustrate some of the experimental data. Figure 6 and Table 1 are

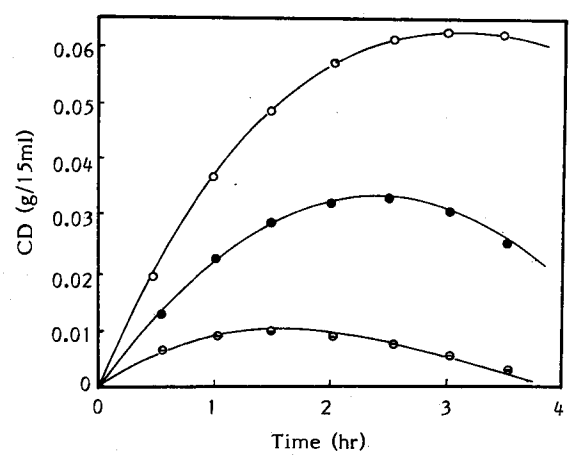

Fig. 5. Formation of CD at different starch concentration in the presence of 0.02 g/ml glucose. Starch concentration wt%=5 (⊖), 7.5 (●), 10 (○).

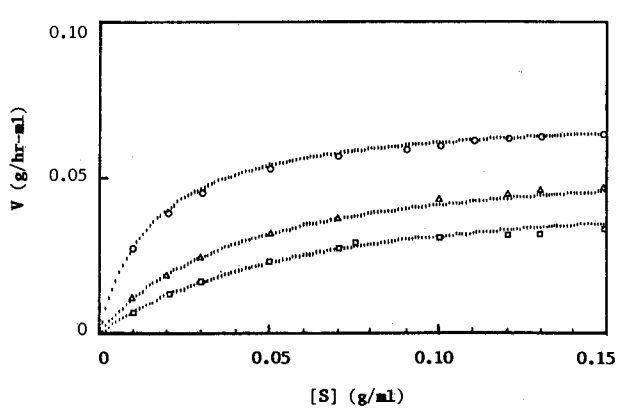

Fig. 6. Plot of V *vs.* S at different glucose concentration. Glucose concentration (g/ml) ○: 0, △: 0.01, □: 0.02. Dot lines are computed from Eq. (7).

Table 1. Glucose effect on the formation of CD

0.01 *g/ml* glucose		0.02 *g/ml* glucose	
[Starch] (*g/ml*)	*V* (*g/hr-ml*)	[Starch] (*g/ml*)	*V* (*g/hr-ml*)
0.01	0.012	0.01	0.007
0.02	0.018	0.02	0.012
0.03	0.025	0.03	0.016
0.05	0.032	0.05	0.023
0.07	0.037	0.07	0.027
0.10	0.043	0.075	0.028
0.12	0.045	0.10	0.0295
0.13	0.046	0.13	0.0315
0.15	0.046	0.15	0.032

the results after using regression analysis. Figure 7 is the plot of $1/V$ *vs.* $1/S$ of Table 1, and the plot suggests that the reaction seemed to be the "linear mixed-type inhibitory reaction". The rate equation of this kinetic model could be expressed as Eq. (5).

$$\frac{1}{V}=\frac{K_s}{V_{max}}\times\left(1+\frac{[I]}{K_i}\right)\times\frac{1}{[S]}+\frac{1}{V_{max}}\times\left(1+\frac{[I]}{a\times K_i}\right) \quad (5)$$

where $[S]$: concentration of starch (*g/ml*), $[I]$: concentration of glucose (*g/ml*).

The parameters of Eq. (5) could be obtained from Fig. 7. Figure 7 shows that the slope of line increased with the concentration of glucose. The dotted vertical line represented the situation where glucose concentration was infinitely large, and this line intercepts the abscissa at $-1/(a\times K_s)$. All the lines cut the ordinate and abscissa at $\{1+[I]/(a\times K_i)\}/V_{max}$ and $-\{1+[I]/(a\times K_i)\}/\{K_s\times(1+[I]/K_i)\}$. It was obtained that $V_{max}=0.0712$ *g* CD/*ml-hr*, $a=10.44$, $K_i=0.07018$ *g/ml*, and $K_s=K_m=0.01623$ *g/ml*. In considering the inhibitory reaction, the formation rate of CD could be expressed as follows:

$$-\frac{dS}{dt}=V=\frac{4.835\times[S]}{1+61.61\times[S]+221.405\times[I]+1389.376\times[S]\times[I]} \quad (6)$$

or

$$-\frac{dS}{dt}=V=\frac{16704.76\times[E_0]\times[S]}{1+61.61\times[S]+221.405\times[I]+1389.376\times[S]\times[I]} \quad (7)$$

where $[E_0]=2.625\times10^{-4}$(*g/ml*), V, V_{max}: *g* CD/*ml-hr*, K_s: *g* starch/*ml*, K_i: *g* glucose/*ml*, a: —.

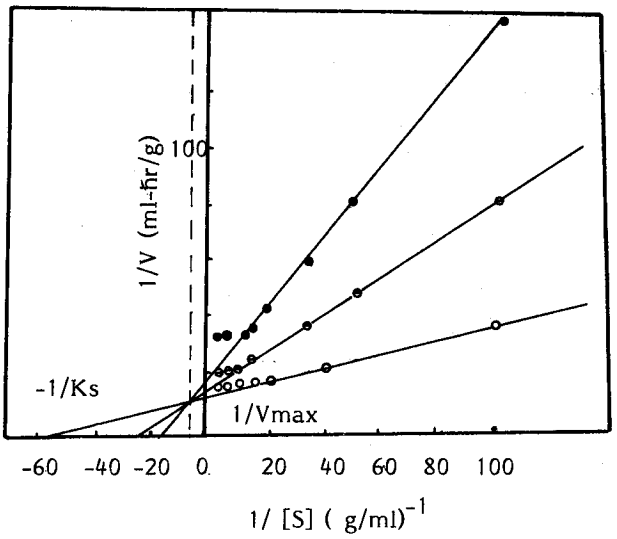

Fig. 7. Lineweaver-Burk plot of Fig. 6. Glucose concentration *g/ml*=0 (○), 0.01 (⊖), 0.02 (●).

The computation of the results of Eq. (7) and experimental observations are shown in Fig. 6.

3. The stability of CD

Experimental results showed that if a lot of glucose was present in the reactant, the yield of CD could not reach an equilibrium quantity. It was also found that the production of CD decreased with time when glucose existed. This is one kind of enzymatic reaction, and its kinetic model is as following:

$$E+P\underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}}EP \qquad ER\underset{K_{-5}}{\overset{K_5}{\rightleftharpoons}}E+R$$

$$+G \qquad +Q$$

$$K_{-2}\uparrow\downarrow K_2 \qquad K_{-4}\uparrow\downarrow K_4$$

$$EPG\underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}}EQR$$

where E: CGTase, P: CD, G: glucose, Q and R: oligosaccharide products (molecular weight smaller than CD), EP, EPG, EQR and ER: intermediates.

Assuming that conversion of EPG to EQR was the rate determining step, then the rate equation would be:

$$\frac{V}{V_{max}}=\frac{[P]\times[G]}{K_aK_g+K_g[P]+[P][G]} \quad (8)$$

where K_a and K_g were equilibrium constants.

The kinetics of the reaction leading to the production of CD will be investigated in the near future.

CONCLUSIONS

The experiment revealed that at low glucose concentrations, the formation rate of CD could be expressed by the Michaelis-Menten equation. When glucose concentration increased during the reaction, its inhibitory effect became notable. After reacting a period of time, the concentration of CD approached a certain constant level. Glucose would not only inhibit the formation of CD but also destroy CD. The degradation reaction was one type of catalytic reaction of this enzyme: the more the glucose, the faster the degradation rate. Thus, in the selection of raw material to produce CD, it would be better to choose a soluble starch suggest: which produces lessglucose during the reaction.

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