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A Consideration on Factors Affecting Synergistic Effect for Hydrolysis of Crystalline Cellulose by Crude Cellulase

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Based on theoretical consideration, the effect of enzyme and substrate concentration on the hydrolysis of crystalline cellulose by cellulase was investigated experimentally. The result suggested that synergistic effect for the hydrolysis of crystalline cellulose, MCC, was closely related to kinetic constants of each cellulase component. From the hydrolysis of MCC by mixed solutions of MCCase and CMCase, which were obtained by partial purification of crude Meicelase, synergistic effect was found to depend on especially Vmax / Km value. In this study, when the amount ratio of MCCase to CMCase was 7 to 1, Vmax / Km value was about 29.4 (μg total sugar / mg protein / min %w/v) and synergistic effect became the highest.

Introduction

It is well known that the effective hydrolysis of crystalline cellulose by cellulases is very difficult. In the hydrolysis of such substrate, the phenomenon called synergistic action occurs, but does not in the hydrolysis of soluble substrate (Nishizawa, 1973). The cause of occurrence of the synergistic action has been investigated mainly on the bases of mechanisms of hydrolysis of crystalline cellulose by cellulases (Okazaki and Moo-Young, 1978)

High synergistic effect in only the case of crystalline cellulose as substrate suggests that the occurrence of synergistic action is closely related to physical structure of the substrate as well as some kinetic constants of each cellulase component in crude cellulase. However, in this study the effect of concentrations of enzyme and substrate on hydrolysis rate of crystalline cellulose was investigated theoretically and experimentally, and also the effect of kinetic constants on synergistic effect was elucidated experimentally.

Theoretical consideration

Crude cellulase preparation is assumed to consist of two cellulase components. The cellulase components, E_1 and E_2, have Michaelis constants, Km_1 and Km_2, respectively.

Initial concentration of sum of the two cellulase components, E_0 is given by eq. (1).

$$E_0 = E_{1,0} + E_{2,0} \quad (1)$$

At an arbitrary reaction time, $E_{1,0}$ and $E_{2,0}$ are given by eqs. (2) and (3), respectively.

$$E_{1,0} = E_1 + E_1S \quad (2)$$

$$E_{2,0} = E_2 + E_2S \quad (3)$$

Where E_1 and E_2 represent the concentrations of free enzymes not adsorbed to substrate, and E_1S and E_2S do the concentrations of enzyme-substrate complexes.

On the other hand, if the concentration of free substrate at an arbitrary reaction time is represented as S, initial concentration of substrate, S_0, is given by eq. (4).

$$S_0 = S + E_1S + E_2S \quad (4)$$

Moreover, Km_1 and Km_2 can be expressed by eqs. (5) and (6), respectively.

$$K_{m1} = E_1 \times S/E_1S \qquad (5)$$

$$K_{m2} = E_2 \times S/E_2S \qquad (6)$$

From eqs. (2) and (5), eq. (7) is obtained.

$$E_1S = E_{1,0}/(K_{m1}/S + 1) \qquad (7)$$

And also, from eqs. (3) and (6), eq. (8) is obtained

$$E_2S = E_{2,0}/(K_{m2}/S + 1) \qquad (8)$$

By substituting eqs. (7) and (8) for eq. (4), S_0 is expressed by eq. (9).

$$S_0 = S + E_{1,0}/(K_{m1}/S + 1) + E_{2,0}/(K_{m2}/S + 1) \qquad (9)$$

Here, if S in eqs. (7) and (8) is small enough, the ratio of E_1S to E_2S is expressed by eq. (10).

$$E_1S/E_2S = (E_{1,0}/E_{2,0})(K_{m2}/K_{m1}) \qquad (10)$$

In general enzyme reaction, the concentration of substrate can be considered to be high enough compared with the concentration of enzyme, but can not in this study, because only cellulose molecules on the surface of crystalline cellulose particles can be used as substrate. Therefore, based on eq. (10), cellulase reaction rate is considered to be influenced by Michaelis constant as well as initial enzyme concentration.

Experimental

Materials

Microcrystallin cellulose No. 2330, MCC, as substrate was purchased from E. Merck A. G. Carboxymethyl cellulose, CMC, was also purchased from Wako Pure Chemical Industries, Ltd. Two kinds of crude cellulase preparation were used for enzyme reaction. One, which was derived from *Trichoderma viride*, was purchased from Worthington Biochemical Corporation. The other, which was also derived from *T. viride*, Meicelase CEPB-5041, was kindly donated by Meiji Seika Ltd.

Methods

Cellulase reaction was carried out using crude cellulase preparation purchased from Worthington Biochemical Corporation to investigate the hydrolysis rate of MCC. The reaction was carried out in 0.05 M sodium acetate buffer (pH 5.0) at 40 °C with shaking. MCC was soaked overnight in the buffer solution before starting enzyme reaction. The concentrations of MCC and the crude cellulase preparation were in the range of 0.25 to 4.0 %w/v and 0.5 to 12 %w/v, respectively. The concentration of total sugar of supernatant in the reaction mixture taken out at intervals and centrifuged was measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956) with glucose as the standard. The initial rates of hydrolysis of MCC ware estimated from the curves of hydrolysis rates obtained under various enzyme and substrate concentrations.

On the other hand, to elucidate the relationship between synergistic effect and kinetic constants of cellulase components, crude Meicelase preparation was partially purified by Amberlite CG-50 (Type II) and DEAE-Sephadex A-50 column chromatographies (Tanaka *et al.*, 1998). The peaks obtained were grouped in two components. CMCase and MCCase, tentatively named here, consisted of peaks FI, FII, and FIII, and peaks FIV and FV, respectively (Tanaka *et al.*, 1988).

Kinetic constants, that is Michaelis constant, Km, and maximum velocity, Vmax, for the hydrolysis of MCC by crude Meicelase, MCCase, CMCase and cellulase solutions of different amount ratios of MCCase to CMCase were obtained by the following experiments. The reaction was carried out in 0.05 M sodium acetate buffer (pH 5.0) at 30 °C for 24 h with shaking. MCC was soaked overnight in the buffer before starting enzyme reaction. The concentrations of MCC were 0.5, 1.0, 2.0, 3.0 and 10.0 %w/v. The concentration of crude Meicelase, MCCase, CMCase and sum of MCCase and CMCase in the solution of an amount ratio of MCCase to CMCase was 0.16 mg protein/ml each. Synergistic effect under different amount ratios of MCCase to CMCase was investigated under MCC concentration of 2.0 %w/v. The synergistic effect was estimated as follows: amount of total sugar produced from MCC by cellulase solution of an amount ratio of MCCase to CMCase were divided by sum of amounts of total sugar produced from MCC by MCCase alone and CMCase alone, which were the same concentrations as in the solution of an amount ratio of MCCase to CMCase, respectively. The amounts of total sugar in the supernatant after 24 h of reaction, that is, the amounts of MCC solubilized by cellulase were measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956) with glucose as the standard. The amount of protein was measured by the method of Lowly *et al.*, 1951 with crystalline bovine serum albumin as the standard. The values of Km and Vmax were estimated from Lineweaver-Burk plots.

Results and discussion

Effect of concentrations of enzyme and substrate on hydrolysis of crystalline cellulose

Ogiwara *et al.*, 1971 have performed the hydrolysis of pulp by cellulase "Onozuka P 500" to elucidate the kinetic characteristics of cellulase during hydrolysis of cellulosic materials. They have reported the followings: as for hydrolysis rate of pulp, an optimal cellulase concentration was present, in other words, the hydrolysis rate of pulp was decreased when the concentration of cellulase was too high: moreover, the optimal cellulase concentration was increased with an increase in the pulp concentration. Fig. 1 shows the relationship between the concentration of crude cellulase and the hydrolysis rate of MCC when a parameter was concentration of MCC. The experimental results were in good agreement with those obtained by Ogiwara et al., 1971 described above. On the other hand, Fig. 2 shows the relationship among E_2S, E_0 and S_0 as a parameter, which were calculated from eqs. (8) and (9) by trial and error method. Where the ratio of cellulase components, $E_{1,0}/E_{2,0}$, and the ratio of Michaelis constants, K_{m1}/K_{m2}, are assumed to be 1 and 1/10, respectively. The curves of the same pattern as in the experimental results were obtained. Therefore, as described in the section of "Theoretical Consideration," when the concentration of substrate is not considered to be high enough, it is obvious that cellulase reaction rate is influenced by Michaelis constant as well as enzyme concentration. However, when Michaelis constants of two cellulase components are considerably different as described above, even if total cellulase concentration becomes high, the amounts of adsorption to substrate of one or the other of two cellulase component to MCC. The hydrolysis rate of substrate depends on synergistic action between cellulase components on the surface of MCC seemed to bring about the decrease in hydrolysis rate of MCC. These theoretical and experimental considerations suggest that synergistic effect is strongly influenced by kinetic constants of each cellulase component.

Effect of kinetic constants of synergistic effect

Km (%w/v) values of crude Meicelase, MCCase and CMCase under the hydrolysis of MCC were 1.1, 0.56 and 6.3, and Vmax (μg total sugar/mg protein/min) values, 17.5, 6.3 and 50.5, respectively. MCCase had the lowest Km value, and CMCase, the highest Vmax value. Km and Vmax values of crude Meicelase were intermediate

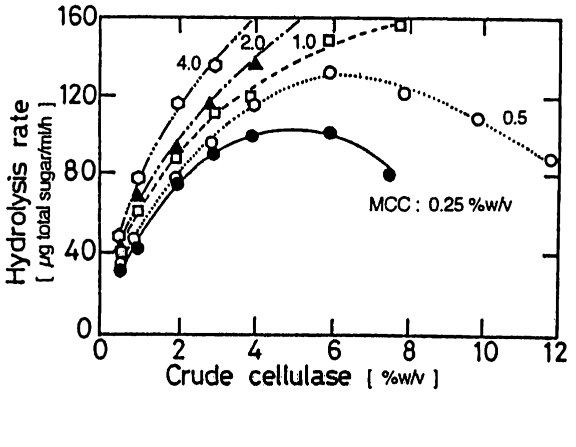

Fig. 1 Relationship between concentration of crude cellulase and hydrolysis rate of MCC when a parameter was concentration of MCC.

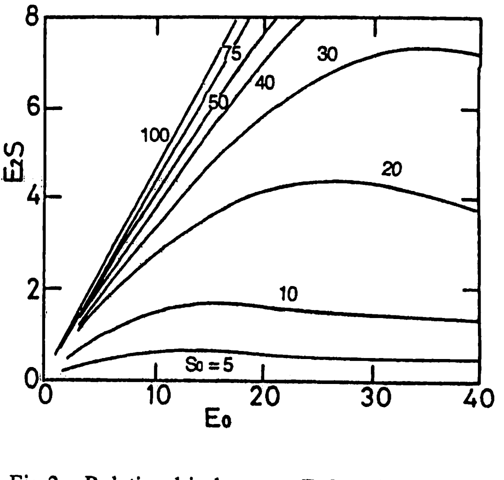

Fig.2 Relationship between E_2S and E_0 on the basis of eqs. (7), (8) and (9) when a parameter was S_0. The ratios of $E_{1,0}$ to $E_{2,0}$ and K_{m1} to K_{m2} are assumed to be 1 and 1/10, respectively.

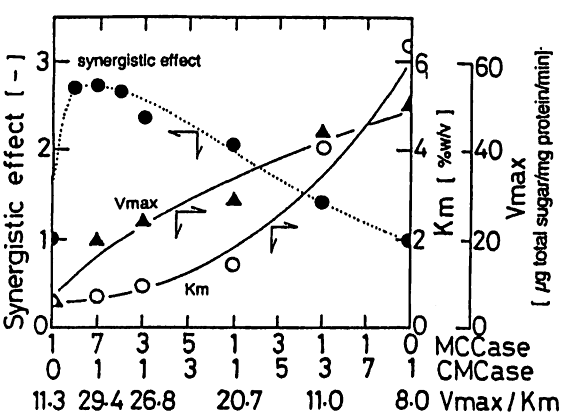

Fig.3. Effect of Km and Vmax values on synergistic effect under various amount ratios of MCCase to CMCase.

between those of two cellulase components obtained from crude Meicelase. Fig. 3 shows the effect of Km and Vmax values of MCCase and CMCase on synergistic effect for the hydrolysis of MCC under various amount ratios of MCCase to CMCase. Km and Vmax values were very different depending on the amount ratios of MCCase and CMCase. When the amount ratio of MCCase to CMCase was approximately 7 to 1, Vmax/Km value become the highest and synergistic effect also became the highest. Vmax and Km values were decreased with an increase in the amount ratio of MCCase to CMCase. However, the degree of decrease in the Km value was remarkable compared with that in Vmax value. These findings indicate that the cause of occurrence of synergistic action existed in the solubilization process of MCC and was closely related to the characteristics of MCCase, which had a high affinity to MCC. This also suggest no synergistic effect in the case of soluble substrate (Nishizawa, 1973). Incidentally, because Vmax/Km values of crude Meicelase was about 15.9 (μg total sugar/mg protein/min%w/v), MCCase and CMCase in crude Meicelase seemed not always to be optimal amount ratio for synergistic effect.

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