KINETICS OF HYDROLYSIS REACTION OF GLYCOL CHITIN WITH EGG WHITE LYSOZYME

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Keywords: Enzymatic Reaction, Lysozyme, Glycol Chitin, Kinetics, Inhibition

Introduction

Chitin, widely found in nature, particularly in marine invertebrates, insects, fungi and algae, is a

biopolymer composed of β -1,4 linked 2-acetamide-2-deoxy-D-glucose (N-acetylglucosamine, (GlcNAc)) residues. Study on effective utilization of chitin is attractive for the fields of wastewater treatment, pharmacology, food industries, surgical instruments, and others. It is of interest that the chitooligosaccharides (degree of polymerization (n) is from 6 to 8)

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degraded from chitin have high physiological activities, that is, activities of elicitors, antibacterial agents, immuno-enhancers, and lysozyme inducers (Aiba, 1994; Deshpande, 1986). Oligosaccharides have been prepared by chemical (Kikkawa *et al.*, 1990; Rupley, 1964), enzymatic (Aiba, 1994, Usui *et al.*, 1990; Yalpani and Pantaleon, 1994) and fermentation (Takiguchi and Shimahara, 1991) methods. In the chemical methods, chitin is degraded by concentrated HCl to form chitinoligosaccharides. Many fragments are formed and the desired fragments must be separated and purified by chromatographic techniques. This method is easy to perform, but the hydrolyzate is mainly composed of monomer and dimer of GlcNAc. The fermentation method is unsuitable for practical use in mass production. In the enzymatic method, chitinoligosaccharides ($n=6-8$) are prepared by the degradation or transglycosilation using a chitinase or lysozyme. Moreover, chito-oligosaccharides can be continuously produced with an immobilized enzyme.

Chitin, which is the substrate in this enzymatic reaction, is essentially insoluble in aqueous and organic media because of the intermolecular and internal hydrogen bond. Accordingly, we prepared a water-soluble chitin derivative, glycol chitin. In water-soluble chitin derivatives, glycol chitin was reported for the substrate of lysozyme and chitinase (Hamaguchi and Funatsu, 1959; Hayashi *et al.*, 1964; Koga *et al.*, 1983; Nakatani and Hiromi, 1974; Ohtakara, 1964; Yamada and Imoto, 1981).

The final goal of this research is establishment of a preparation system of physiologically active chito-oligosaccharides with enzymatic reactions, such as hydrolysis and transglycosilation with chitinase or lysozyme. So as basic research, the kinetics of hydrolysis of glycol chitin by lysozyme was investigated. The information obtained here will be extended to a system of formation of physiologically active chito-oligosaccharides with chitinase and lysozyme.

1. Experimental

1.1 Substrate

As the substrate of lysozyme, the water-soluble chitin derivative, glycol chitin, was synthesized according to the following reaction scheme (Yamada and Imoto, 1981).

Fig. 1 pH Dependence of lysozyme activity ($T=310\text{ K}$, $t=120\text{ min}$)

The degree of substitution could be calculated from the content of C and N as found by elemental analysis. As a result, it was estimated to be 0.617. The average molecular weight of the glycol chitin was determined as about 24000 from measuring by viscometry.

1.2 Enzymatic reactions

GR grade reagent, lysozyme chloride from hen egg white, was purchased from Nacalai Tesque Inc. This was used as the enzyme without further purification. The substrate solution was prepared by mixing the glycol chitin and citric acid-disodium hydrogen phosphate buffer solution. Enzymatic reaction was initiated by adding 1 ml of the enzyme aqueous solution with a concentration of 0.1 g/dm^3 into 7 ml of the substrate solution with a desired concentration (concentration of lysozyme resulted in $1.25 \times 10^{-5}\text{ kg/m}^3$). Firstly the reaction was carried out at 310 K. After an appropriate time, the reaction was terminated by adding the deproteinizing agent, sodium tungstate, dissolved in diluted sulfuric acid. The precipitates were removed by centrifugation. The degree of reaction was followed by the modified Schales method. (Imoto and Yagishita, 1971). Here the reaction rate was identified as the rate of GlcNAc or $(\text{GlcNAc})_2$ formation. So the reaction rate was obtained by analysis of reducing sugar of GlcNAc formed in this hydrolysis reaction. The analysis of the reducing sugar was conducted using potassium ferricyanide, that is the enzyme activity was evaluated by measuring the change in absorbance at 418.5 nm during the reduction of Fe(III) to the Fe(II) with aldehyde group in the reducing sugar.

The effects of pH, the substrate concentration and reaction temperature on the hydrolysis reaction were examined to clarify the enzymatic reaction mechanism. Furthermore, the effects of metal ions, K^+ , Mg^{2+} , Ca^{2+} , and Al^{3+} , on the reaction were also examined. The concentrations of each metal ion in the reaction media were 11.1 mol/m^3 .

Fig. 2 Lineweaver-Burk plot ($T=310$ K, pH 4.20). Slope = $1.65 \times 10^4 \pm 8.42 \times 10^3$ s. Intercept at longitudinal axis = $5.55 \times 10^3 \pm 3.46 \times 10^3$ Correlation coefficient = 0.964

Fig. 3 Arrhenius plot for the reaction of lysozyme with glycol chitin (pH 4.20)

2. Results and Discussion

2.1 Kinetics

The pH dependence of this enzymatic reaction was shown in Fig. 1, where the optimum pH is in the range from 4 to 5. Moreover, the details of the pH dependence were investigated, which indicated the optimum pH to be 4.2. Hamaguchi and Funatsu (1959) reported that the optimum pH of lysozyme were 3.5 and 5 to 7.4 for the hydrolysis of glycol chitin, and for the lysis of bacteria, respectively. In this work the optimum pH was close to the former value, 3.5. The following experiments were carried out at pH=4.2. From the Lineweaver-Burk plot shown in Fig. 2, the maximum velocity, $V_{\max}$, and the Michaelis constant, K_m , were obtained as 1.80×10^{-4} kg/(m³ s) (ranging from 1.11×10^{-4} to 4.78×10^{-4} kg/(m³ s)), and 2.95 kg/m³ (ranging from 2.77 to 3.87 kg/m³), respectively. In our experiment, it is difficult to use the same enzyme and substrate. From an Arrhenius plot of this enzymatic reaction as shown in Fig. 3, the apparent activation energy, E_a , was determined as

Fig. 4 Dependence of metal ions on reactivity of lysozyme ($T=310$ K, pH 4.20)

Fig. 5 Lineweaver-Burk plot in presence of aluminum ion ($T=310$ K, pH 4.20)

98.9 kJ/mol. This was larger than those of the other enzyme reactions (Thomas, 1980).

2.2 Effect of metal ions

The effect of adding the metal ions on the enzymatic reaction were shown in Fig. 4. It was found that all of the metals inhibited this enzymatic reaction. The magnitude of inhibition was increased according to the order, $\text{Ca}^{2+} < \text{K}^+ < \text{Mg}^{2+} < \text{Al}^{3+}$. Particularly Al^{3+} most inhibited this reaction, and the quantity of the product formed at 180 min was about 50% quantity when the metal ions were not added. It is anticipated that there is some interaction between Al^{3+} and the active site of lysozyme concerning the relationship between both sizes. To find out the inhibition mechanism of Al^{3+} on this hydrolysis reaction, the Lineweaver-Burk plots in the cases for which Al^{3+} was or was not added are shown in Fig. 5. Two fitted straight lines nearly crossed at a point on the $1/v_0$ axis. So it is found that the inhibition mechanism of Al^{3+} in this reaction is competitive inhibition expressed by Eq. (1).

$$\frac{1}{v_0} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} \quad (1)$$

The inhibition constant, K_i , was determined as 0.414 kg/m³ from Eq. (1) and Fig. 5.

Conclusion

The findings of this experimental work were as follows. (1) This enzymatic reaction was conformed to be followed by Michaelis-Menten type kinetics from linear fitting of the Lineweaver-Burk plot, and the maximum velocity and the Michaelis constant were determined. (2) From the Arrhenius plot, the apparent activation energy of this enzymatic reaction was estimated as 98.9 kJ/mol. (3) As the result of investigating the effects of some metal ions on the reactivity of lysozyme, all metal ions studied here, K⁺, Ca²⁺, Mg²⁺, and Al³⁺, acted as inhibitors. The effect of Al³⁺ was particularly remarkable. The inhibition mechanism was found to be competitive, and the inhibition constant was determined as 0.414 kg/m³.

Nomenclature

E_a	= apparent activation energy	[kJ/mol]
K_i	= inhibition constant	[kg/m ³]
K_m	= Michaelis constant	[kg/m ³]
t	= reaction time	[s]
$V_{\max}$	= maximum velocity	[kg/(m ³ s)]
v_0	= initial reaction rate	[kg/(m ³ s)]
[I]	= concentration of inhibitor	[kg/m ³]
[S]	= concentration of substrate	[kg/m ³]

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