Kinetic Analysis of Enzymatic Hydrolysis of Raw Starch by Glucoamylase Using an Amperometric Glucose Sensor

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An amperometric glucose sensor has been introduced to study the kinetics of enzymatic hydrolysis of raw starch by glucoamylase in a thick raw starch suspension. The initial rate of the hydrolysis increased with increasing amount of enzyme to approach a saturation value, whereas it was proportional to the amount of substrate. The dependence can be explained by the rate equation based on the Langmuir adsorption isotherm.

Much attention has been paid to enzymatic hydrolysis of raw starch by α-amylase,[1–3] β-amylase,[4] and glucoamylase.[5–10] In most studies, the reaction has been followed by spectrophotometry of saccharides in the reaction mixture after removing raw starch at different times. In comparison with spectrophotometry, electrochemical measurements have advantages of being free from the influence of turbidity and coloration of a test solution. Electrochemical sensors have been applied to analysis of turbid samples such as blood,[11] foods,[11,12] and bacterial cell suspensions.[13–15] In this study, an amperometric glucose sensor, in which the oxidation current of the redox mediator reduced by the glucose oxidase (GOx) reaction is monitored, has been introduced to study the kinetics of enzymatic hydrolysis of raw starch by glucoamylase (EC 3.2.1.3). Direct and continuous observation of the hydrolysis in a thick raw starch suspension can be achieved. The dependence of the initial rate of the hydrolysis on the amount of enzyme and substrate is discussed.

Glucoamylase from *Rhizopus* sp. was obtained from Toyobo and used as received. The bulk concentration of the free, or unbound, enzyme in a raw starch suspension, $[E_f]$, was determined from the ultraviolet absorbance at 280 nm ($\varepsilon = 1.0 \times 10^5\ M^{-1}\cdot cm^{-1}$)[16] after centrifugation. Raw starch granules from corn were obtained from Wako. The granule diameter is in the range of 5–25 μm.[17] They were washed twice with distilled water and dried before use. A 0.025–1.0 g of dry raw starch was suspended in 5.0 mL of 0.1 M (M = mol·dm^{-3}) acetate buffer (pH 5.0) for the electrochemical measurement. A film-coated GOx-immobilized benzoquinone (BQ)-mixed carbon paste electrode (CPE) was prepared as described by Ikeda et al.[18] A 30 U of GOx (Sigma) was trapped by a dialysis membrane (20-μm thick in the dry state) on the surface of CPE (3.0-mm diameter) containing 10 wt % of BQ in carbon paste. An oxidation current was recorded at a fixed electrode potential of 0.60 V vs Ag|AgCl| 0.1 M KCl without deaeration. The suspension was stirred by a magnetic stirrer at 500 rpm. The film-coated GOx-immobilized BQ-mixed CPE allowed the determination of D-glucose concentration in the suspension, [Glc], sensitive to levels as low as 0.01 mM, with a linearity of up to 10 mM. The sensitivity of the electrode was $0.39 \pm 0.02\ \mu A\cdot mM^{-1}$. The response time was approximately 20 s. The experiments were performed at $25 \pm 0.5\ ^\circ C$.

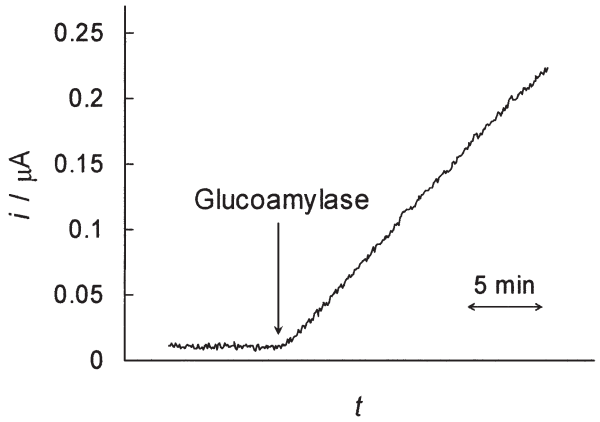

Figure 1. Current (i)–time (t) curve for the production of glucose. Measurement was carried out in a raw starch suspension ($S = 0.010\ g\cdot cm^{-3}$) at pH 5.0. Glucoamylase ($[E_f] = 0.4\ \mu M$) was added at the point indicated by the arrow.

Figure 1 shows an example of the current response to the production of D-glucose in the raw starch suspension. The amount of raw starch, S, was $0.010\ g\cdot cm^{-3}$. Before the addition of glucoamylase to the suspension, the current was so small that the contamination of glucose from the washed raw starch can be neglected. By the addition of glucoamylase ($[E_f] = 0.4\ \mu M$), the current began to increase linearly. The initial slope of the current–time curve was used to determine the initial rate, v, for the production of glucose. Interestingly, the v value increased with increasing $[E_f]$ to approach a saturation value (Figure 2) at $S = 0.020\ g\cdot cm^{-3}$, whereas it was proportional to S in the range between 0.005 and $0.20\ g\cdot cm^{-3}$ (Figure 3) at $[E_f] = 0.4\ \mu M$. On the contrary, when boiled soluble starch was used in place of raw starch, the v value was proportional to the total enzyme concentration and showed a saturation tendency with the concentration of soluble starch in accordance with the familiar Michaelis–Menten equation.

It is known that the raw-starch-digesting glucoamylases adsorb onto the surface of raw starch granules.[5–10] This adsorption is believed to be the initial step of the hydrolysis:[6–8]

$$E_f + (Glc)_n = E_{ad} \cdot (Glc)_n \quad (1a)$$

$$E_{ad} \cdot (Glc)_n + H_2O \rightarrow E_f + (Glc)_{n-1} + Glc \quad (1b)$$

where E_f and E_{ad} are the free and adsorbed enzyme, respectively. If we assume that the fractional coverage of the binding sites of raw starch with the enzyme (θ) can be expressed by the Langmuir adsorption isotherm and that the rate of Eq 1b is proportional to θ and the number of the binding sites available, which is approximately proportional to S, we obtain the following rate equation:

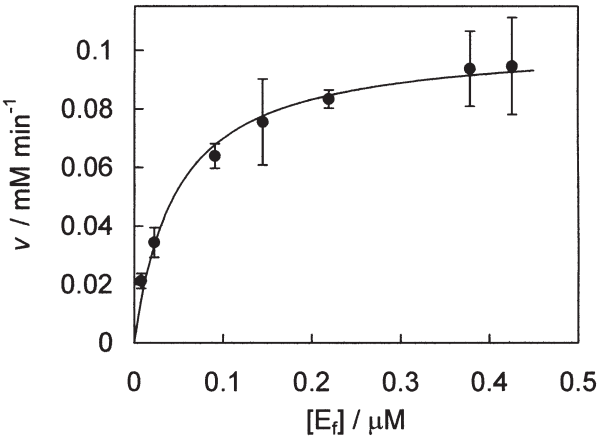

Figure 2. Dependence of v on $[E_f]$. $S = 0.020\,g{\cdot}cm^{-3}$. Solid line is calculated by Eq 2 with the k and K_{ad} values given in the text. The vertical bars indicate the standard deviations.

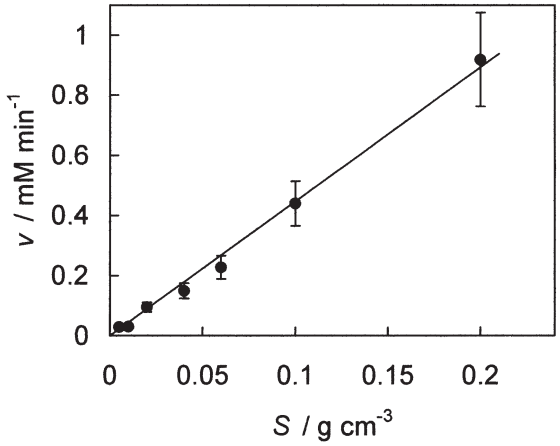

Figure 3. Dependence of v on S. $[E_f] = 0.4\,\mu M$. Solid line is calculated by Eq 2 with the k and K_{ad} values given in the text. The vertical bars indicate the standard deviations.

$$v = \frac{d[Glc]}{dt} = kS\theta = \frac{kSK_{ad}[E_f]}{1 + K_{ad}[E_f]} \quad (2)$$

where k and K_{ad} are the rate constant (see below) and the adsorption coefficient, respectively. The dependence of v on $[E_f]$ and on S shown in Figures 2 and 3, respectively, are consistent with the prediction from Eq 2. The two parameters k and K_{ad} were determined to be $5.0\,\mu mol{\cdot}g^{-1}{\cdot}min^{-1}$ and $2.1 \times 10^7\,M^{-1}$, respectively, by fitting Eq 2 to the experimental data in Figure 2.

The rate constant k can be given by:

$$k = n_W k_0 \quad (3)$$

where n_W is the number of binding site per unit weight of raw starch and k_0 is the turnover number. Using $n_W = 7.0 \times 10^{-9}$ $mol{\cdot}g^{-1}$ determined from the difference of the amounts between the total and free enzymes at $[E_f] = 0.4\,\mu M$, k_0 was calculated to be $1.2 \times 10\,s^{-1}$, which is comparable to $24\,s^{-1}$ for soluble amylose hydrolysis by glucoamylase.[16]

Enzymatic hydrolysis of microcrystalline cellulose (Avicel®) by the crude extract of cellulase from *Trichoderma viride* has also been studied by the present method. The dependence of v on $[E_f]$ and on S were similar to those of the raw starch hydrolysis. The k and K_{ad} values were determined tentatively to be $0.42\,\mu mol{\cdot}g^{-1}{\cdot}min^{-1}$ and $1.8\,mg^{-1}{\cdot}cm^3$, respectively, at 40 °C. It is interesting to apply the present electrochemical method for the quantitative kinetic studies of the hydrolysis of raw starch and cellulose as well as other insoluble substrates.

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