JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Kinetics of the Surface Hydrolysis of Raw Starch by Glucoamylase

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The hydrolysis of raw starch catalyzed by glucoamylase has been studied with starch granules of different sizes by use of an amperometric glucose sensor by which the direct and continuous observation of the concentration of glucose can be achieved even in a thick raw starch suspension. The initial rate of the enzymatic hydrolysis in the raw starch suspension increased with increasing concentration of the enzyme to approach a saturation value and was proportional to the amount of substrate. Also, the rate was proportional to the specific surface area of the substrate. The experimental results can be explained well by the rate equations derived from a three-step mechanism, which consists of adsorption of the free enzyme onto the surface of the substrate, reaction of the adsorbed enzyme with the substrate, and liberation of the product.

KEYWORDS: Interfacial enzymes; surface hydrolysis; glucoamylase; raw starch; Langmuir adsorption isotherm; biosensor

INTRODUCTION

Glucoamylase (EC 3.2.1.3) is an exo-acting glycoside hydrolase that produces D-glucose from the nonreducing ends of starch. In recent years some glucoamylases capable of direct hydrolysis of raw starch have received considerable attention as their potential value for certain biotechnological applications, such as the production of ethanol (*1*−*8*) or lactic acid (*9*) from a starchy biomass, was recognized.

Although many reports have appeared on enzymatic hydrolysis of raw starch by glucoamylase (*10*−*19*), quantitative kinetic analyses of the hydrolysis have not been provided. In our previous report (*20*) it was shown that direct and continuous observation of the hydrolysis in a thick raw starch suspension can be achieved by use of an electrochemical glucose sensor. Also, it was shown that the initial rate of the hydrolysis of raw corn starch increased with the increasing amount of glucoamylase to approach a saturation value, whereas it was proportional to the amount of starch.

In the present work the kinetic study has been extended to the hydrolysis of starch granules of different sizes, aiming to elucidate the dependence of the rate on the surface area of the substrate and on the adsorbed amount of enzyme. The experimental results can be explained by the rate equations derived from a three-step mechanism, which consists of adsorption of the free enzyme onto the surface of the substrate (*10*−*19*), reaction of the adsorbed enzyme with the substrate, and liberation of the product. The results and discussion are given in the paper.

THEORETICAL BASIS

We consider the following model in accordance with lipolysis (*21*−*23*)

$$E_f \rightleftarrows E_{ad} \tag{1a}$$

$$E_{ad} + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_{ad}S \tag{1b}$$

$$E_{ad}S \xrightarrow{k_2} E_{ad} + P \tag{1c}$$

that is, the free, or unbound, glucoamylase (E_f) is adsorbed onto the surface of the substrate according to eq 1a, then the adsorbed enzyme (E_{ad}) is bound with the substrate (S, here, the nonreducing end of starch) to give a productive complex ($E_{ad}S$) according to eq 1b, which is followed by liberation of the product (P, here, glucose) according to eq 1c. It should be noted that a so-called nonproductive complex (*24*) is included by E_{ad} for simplicity.

We assume the adsorption and desorption of eq 1a are very fast processes so that the Langmuir adsorption isotherm (*25*−*28*) is valid with respect to the concentration of E_f ($[E_f]$)

$$\Gamma/\Gamma_{max} = \beta[E_f]/(1 + \beta[E_f]) \tag{2}$$

where $\Gamma = \Gamma_{Ead} + \Gamma_{EadS}$, Γ_{Ead} and Γ_{EadS} are the adsorbed amount of E_{ad} and $E_{ad}S$ per unit area, respectively, Γ_{max} is the saturation value of Γ, and β is the adsorption coefficient. The rate equations for eqs 1b and 1c are given by, respectively

$$d\Gamma_{EadS}/dt = k_1'\Gamma_{Ead} - (k_{-1} + k_2)\Gamma_{EadS} \tag{3}$$

and

$$d[P]/dt = k_2\Gamma_{EadS}(A/V) \tag{4}$$

where [P] is the concentration of P, A the surface area of the substrate, and V the volume of the test solution. In the derivation

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10.1021/jf050934c CCC: $30.25
Published on Web 09/16/2005

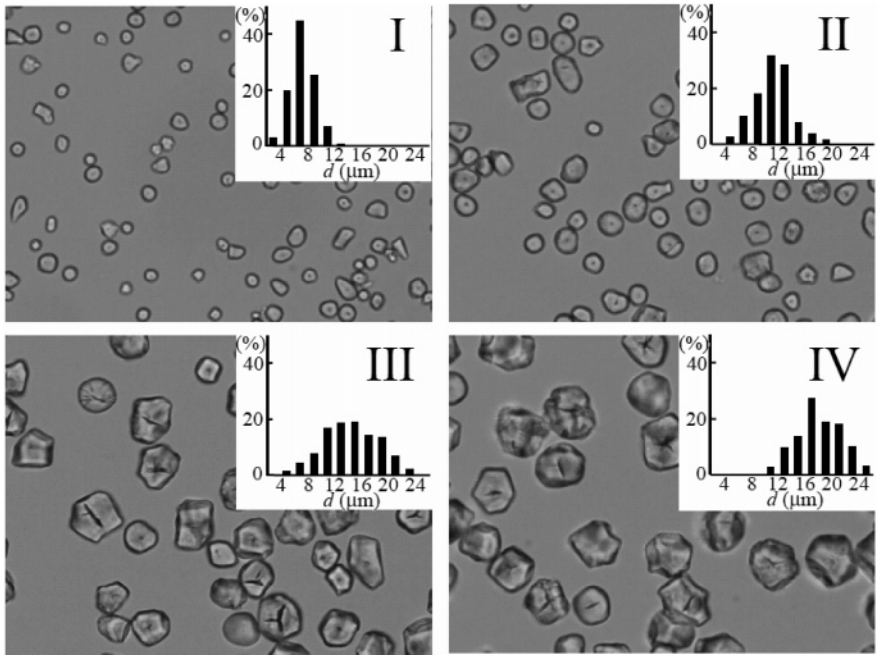

Figure 1. Microscopic images of fractions I, II, III, and IV. The scale of each picture is 170 μm × 130 μm. The insets show the size distribution for each fraction.

of eq 3 the pseudo-first-order reaction with respect to S is assumed to be satisfied in the forward step of eq 1b because the surface density of the nonreducing end of starch (Γ_S) does not change at the initial stage of the reaction, so that the pseudo-first-order rate constant (k_1') is used here and in the following.

At steady state the rate (v) is given by

$$v = \mathrm{d[P]/d}t = v_{max}\beta[E_f]/(1 + \beta[E_f]) \quad (5a)$$

with

$$v_{max} = k_0\Gamma_{max}(A/V) \quad (5b)$$

where k_0 represents the molecular activity of the adsorbed enzyme, as given by

$$k_0 = k_2/(1 + K_m') \quad (5c)$$

with

$$K_m' = (k_{-1} + k_2)/k_1' \quad (5d)$$

The dimensionless parameter K_m' in eq 5d can be equated to the ratio of Γ_{Ead} to Γ_{EadS} at the steady state. The term A/V in eqs 4 and 5b is related to the specific surface area of the substrate (a) and the weight of the substrate per volume (S) by $A/V = aS$, so that v_{max} is also given by

$$v_{max} = k_0\Gamma_{max}aS \quad (6)$$

Assuming that starch granules are spheres with a constant density (ρ) and with various diameters (d), the a value can be related to ρ and d by

$$a = (6/\rho)(\Sigma d^2/\Sigma d^3) \quad (7)$$

where Σd^2 and Σd^3 are the sum of d^2 and d^3, respectively, of a given amount of starch granules.

MATERIALS AND METHODS

Materials. Raw starch granules from corn were obtained from Wako. The native raw starch granules (5−25 μm diameter) were separated into four fractions according to their sizes as follows: 2 g of the native raw starch granules was suspended in 200 mL of distilled water and centrifuged at 5*g* for 10 min. The granules in the supernatant (fraction I) were collected. The precipitate was then suspended in 200 mL of distilled water and centrifuged at 5*g* for 2 min. The granules in the supernatant (fraction II) were collected. The precipitate was suspended in 200 mL of distilled water and gently stirred in a 250 mL cylinder. After standing for 10 min, the granules in the supernatant (fraction III) and the precipitate (fraction IV) were collected. We obtained 0.06 g of fraction I, 0.31 g fraction of II, 0.93 g of fraction III, and 0.55 g of fraction IV from 2 g of the native raw starch. **Figure 1** shows the microscopic images of fractions I, II, III, and IV. The insets show the size distribution determined by measurements of about 200 granules for each fraction from which the a values were calculated by eq 7 with $\rho = 1.42$ g cm^{-3} obtained by a pycnometer. Each fraction was dried in a desiccator for several days before use. A 0.025−0.25 g amount of the fraction was suspended in 5.0 mL of 0.1 M buffer solution (pH 3.0−7.0) for the electrochemical measurement.

Glucoamylase (GA; Glucan 1,4-α-glucosidase from *Rhizopus* sp., 38.5 U mg^{-1}) was obtained from Toyobo and used as received. The bulk concentration of the free GA in a raw starch suspension, $[E_f]$, was determined from the ultraviolet absorbance at 280 nm, assuming the absorbance of 1% solution to be 14.5 cm^{-1} and a molecular weight of 70 000 (*24*), that is, a molar extinction coefficient (ϵ) of 1.0 × 10^5 M^{-1} cm^{-1} (M = mol dm^{-3}), after removing the starch granules by centrifugation. The adsorbed amount of GA on the substrate surface, Γ, was determined from the decrease of the enzyme concentration by the addition of raw starch granules (*10*, *11*, *14*−*16*). The Γ value can be expressed as

$$\Gamma = ([E]_0 - [E_f])/aS \quad (8)$$

where $[E]_0$ is the total concentration of GA added.

Soluble starch was obtained from Merck and used after boiling at 90 °C for 10 min. Other chemicals were of reagent grade and used as received.

Preparation of an Amperometric Glucose Sensor. A glucose oxidase-immobilized benzoquinone-mixed carbon paste electrode was prepared as described by Ikeda et al. (*29*,*30*). In this study graphite powder containing 25 wt % liquid paraffin and 8 wt % *p*-benzoquinone was packed into a carbon paste electrode holder (BAS Inc., no. 002210). A 10-μL aliquot of 3 U μL^{-1} glucose oxidase (Sigma) solution was dropped onto the surface of the benzoquinone-mixed carbon paste electrode of geometrical area 0.071 cm^2. The solvent was allowed to

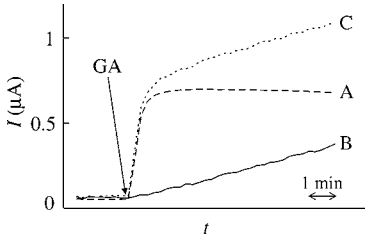

Figure 2. Current (I)–time (t) curve for the production of glucose. Measurements were carried out in 0.1 M acetate buffer (pH 5.0) containing (**A**) 0.25 mg cm^{-3} soluble starch, (**B**) 0.020 g cm^{-3} raw starch (fraction IV), or (**C**) both. GA (2 U mL^{-1}) was added at the point indicated by the arrow. The sensitivity of the glucose sensor was 0.47 μA mM^{-1}.

evaporate, and then the electrode surface was covered with a dialysis membrane (Viskase Inc., cutoff molecular weight of 12 000−14 000, 20-μm thick in the dry state). The electrode was covered by a nylon net to give it physical strength.

Electrochemical Measurements. The concentration of D-glucose, [Glc], was followed amperometrically using the glucose sensor. The electrode potential was fixed at +0.60 V vs Ag|AgCl|0.1 M KCl reference electrode, and the current (I) due to the glucose oxidase-catalyzed oxidation of glucose was recorded as a function of time (t). The test solution was stirred by a magnetic stirrer at 500 rpm, at which the concentration polarization of glucose and the sedimentation of the starch granules can be neglected. The glucose sensor allowed determination of [Glc] sensitive to levels as low as 0.01 mM with a linearity of up to 10 mM without deaeration of the test solution. The response time was approximately 20 s. The experiments were performed at 25 ± 0.5 °C.

RESULTS AND DISCUSSION

Curve A of **Figure 2** shows the $I-t$ curve for the production of glucose in a 0.25 mg cm^{-3} boiled soluble starch solution (pH 5.0). Before addition of GA to the solution the current was so small that contamination of glucose from starch can be neglected. Upon addition of 2 U mL^{-1} GA the current began to increase due to production of glucose, and it reached a constant value within a couple of minutes. From the constant current the final concentration of glucose was determined to be 1.5 mM, which agreed with the calculated value for the complete hydrolysis of 0.25 mg cm^{-3} starch. Curve B of **Figure 2** shows the $I-t$ curve obtained with a raw starch suspension of fraction IV of S = 0.020 g cm^{-3} (pH 5.0). Upon addition of 2 U mL^{-1} GA the current increased linearly. When the supernatant was used for the measurement in place of the suspension, the current did not change by addition of GA. The results indicate that dissolution of the raw starch into the bulk solution was negligible and that the current increase of curve B can be attributed to production of glucose by the surface hydrolysis of raw starch by GA. It should be noted that a steady state of the hydrolysis was attained immediately after addition of GA, and a lag period, which has been reported for lipolysis (*21*,*22*,*28*,*31*), was not observed here. Curve C of **Figure 2** shows the $I-t$ curve when both soluble starch (0.25 mg cm^{-3}) and raw starch (0.020 g cm^{-3}) were used. Upon addition of GA the current increased in the same way as curve A and then linearly with the same slope as curve B, indicating that the rate (v) of the surface hydrolysis of raw starch by GA can be determined from the slope of the linear increase of the current after the inflection point even if the suspension contains soluble starch.

Figure 3A shows the plot of the v value vs [E_f] obtained with the raw starch suspension of fraction IV of S = 0.010 g cm^{-3}. The v value increased with increasing [E_f] to approach a saturation value, which is consistent with the prediction from

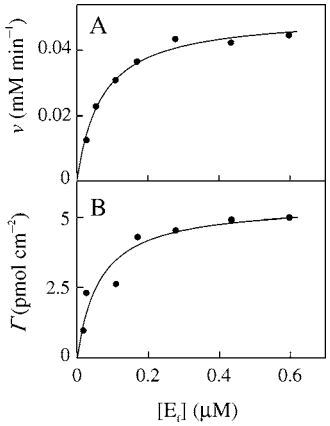

Figure 3. (**A**) Dependence of v on [E_f] obtained with fraction IV of S = 0.010 g cm^{-3}. Solid line is calculated by eq 5a using the β and v_{max} values given in the text. (**B**) Adsorption isotherm of GA obtained with fraction IV of S = 0.010 g cm^{-3}. Solid line is calculated by eq 2 using the β and Γ_{max} values given in the text.

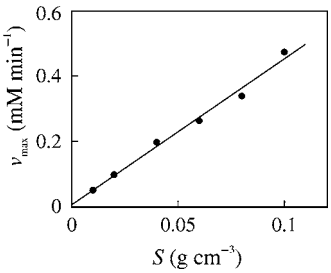

Figure 4. Dependence of v_{max} on S with fraction IV ($a = 2.2 \times 10^3$ cm^2 g^{-1}).

eq 5a. In **Figure 3A** the solid line is the fitted curve by eq 5a, giving $\beta = (1.4 \pm 0.4) \times 10^7$ M^{-1} and $v_{max} = 0.051 \pm 0.005$ mM min^{-1}.

In view of eqs 2 and 5a the same dependence on [E_f] can be expected for the Γ value. **Figure 3B** shows the plot of the Γ value calculated by eq 8 with the determined $[E]_0 - [E_f]$ and a values. The Γ value increased with increasing [E_f] to approach a saturation value. In **Figure 3B** the solid line is the fitted curve by eq 2, giving $\beta = (1.6 \pm 1.3) \times 10^7$ M^{-1} and $\Gamma_{max} = 5.5 \pm 1.3$ pmol cm^{-2}. The β values obtained from **Figure 3A** and **3B** are in good agreement with each other. The Γ_{max} value may be compared with the calculated value of 5 pmol cm^{-2} assuming that GA molecules are spheres with a radius of 3 nm and form a closest-packed monolayer on the substrate surface, suggesting that the surface of raw starch granules is fully covered with GA molecules when $[E_f] \gg \beta^{-1}$ and that the saturation of v by [E_f] is due to the steric hindrance by GA itself. Studies including evaluation of the Γ_S value and comparison with the determined Γ_{max} value are under way in our laboratory.

Figure 4 shows the plot of the v_{max} value vs S obtained by measurement at $[E_f] \gg \beta^{-1}$ with fraction IV. The v_{max} value was proportional to S in the range tested, which is consistent with the prediction from eq 6. The regression line in **Figure 4** is expressed by

$$v_{max}\,(\text{mM min}^{-1}) = (4.5 \pm 0.6) \times S\,(\text{g cm}^{-3}) + (0.01 \pm 0.04) \quad (9)$$

Using eq 6 with $\Gamma_{max} = 5.5 \pm 1.3$ pmol cm^{-2} and $a = 2.2 \times 10^3$ cm^2 g^{-1} for fraction IV (see below), k_0 is calculated to be 6.3 ± 1.7 s^{-1}.

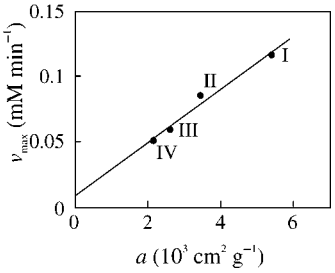

Figure 5. Dependence of v_{max} on a when $S = 0.010$ g cm^{-3}. The fraction numbers (I, II, III, and IV) are indicated beside the circles.

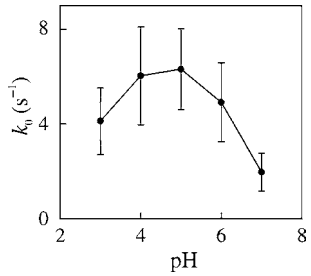

Figure 6. Dependence of k_0 on pH. Measurements were carried out in 0.1 M glycine buffer (pH 3.0), 0.1 M acetate buffer (pH 4.0 and 5.0), or 0.1 M phosphate buffer (pH 6.0 and 7.0). The vertical bars indicate the 95% confidence intervals ($n = 6$).

The dependence of v on $[E_f]$ was also examined with fractions I, II, and III of $S = 0.010$ g cm^{-3}. A dependence similar to **Figure 3A** was observed for each fraction. The v_{max} values were determined to be 0.116, 0.085, and 0.059 mM min^{-1} for fractions I, II, and III of $S = 0.010$ g cm^{-3}, respectively. In **Figure 5** the v_{max} values are plotted vs the a values, which are calculated to be 5.4×10^3, 3.4×10^3, 2.6×10^3, and 2.2×10^3 cm^2 g^{-1} for fractions I, II, III, and IV, respectively, using eq 7 with the determined ρ and d values (see Materials and Methods). The v_{max} value was proportional to a, which is consistent with the prediction from eq 6. The regression line in **Figure 5** is expressed by

$$v_{max}\,(\text{mM min}^{-1}) = \{(2.0 \pm 0.9) \times 10^{-5}\} \times a\,(\text{cm}^2\,\text{g}^{-1}) + (0.01 \pm 0.03) \quad (10)$$

Using eq 6 with $\Gamma_{max} = 5.5 \pm 1.3$ pmol cm^{-2} and $S = 0.010$ g cm^{-3}, k_0 is calculated to be 6.1 ± 3.1 s^{-1}, which agrees with 6.3 ± 1.7 s^{-1} obtained above. These k_0 values appear to be somewhat smaller than 24 ± 1 s^{-1} reported by Hiromi et al. as the molecular activity of GA for the hydrolysis of maltodextrin (average degree of polymerization of 15.5) at pH 4.5 (*24*), suggesting that insoluble substrate is to some extent unfavorable for the formation of a productive complex as compared with soluble substrate, probably due to the inaccessibility of the nonreducing end of starch to the catalytic site of GA. If we take, for example, that $k_0 = 6.2$ s^{-1} on average and that $k_2 = 77$ s^{-1} reported by Hiromi et al. as the intrinsic rate constant of GA for the hydrolysis of substrate linkage in a productive complex (*24*), we obtain $K_m' = 11$ from eq 5c. This means that 1 of 12 adsorbed GA molecules forms a productive complex at the steady state (see Theoretical Basis).

Figure 6 shows the effect of pH on the k_0 value examined with fraction IV of $S = 0.010$ g cm^{-3}. The dependence of k_0 on pH was similar to that of soluble starch hydrolysis with the pH optima in the range of 4.5−5.0 (*32*). No significant change was observed in Γ_{max} and β over the pH range between 3.0 and 7.0.

The above results clearly show that the rate law of the hydrolysis of raw starch by GA follows the present kinetic model of eqs 1a−c. Now the study is being extended to starch granules from other plants as well as glucoamylases from other microorganisms and extraction of the kinetic parameters of their reactions.

ACKNOWLEDGMENT

We are grateful to Noriharu Maeda and Satsuki Takabe for assistance in experiments.

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Received for review April 22, 2005. Revised manuscript received July 31, 2005. Accepted August 18, 2005. This work was supported by a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology, Japan (16780086, to H.T.).

JF050934C