Different Behavior towards Raw Starch of Two Glucoamylases from *Aspergillus saitoi*

Tomoko TAKAHASHI,*[,a] Yoshio IKEGAMI,[b] Masachika IRIE[b] and Etsuko NAKAO[a]

Institute of Medicinal Chemistry[a] and Faculty of Pharmaceutical Sciences,[b] Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan.
Received April 26, 1990

Two glucoamylases [EC 3.2.1.3] of *Asperigillus saitoi*, Gluc M_1 (molecular weight (M.W.) 90000) and Gluc M_2 (M.W. 70000), which have similar pH optima and specific activities towards soluble starch were studied as to their behavior towards raw starch. The pH optima for raw starch digestion were different, 3.5 for Gluc M_1 and 4.0 for Gluc M_2. These enzymes digested raw starch almost completely but at quite different rates, Gluc M_2 being 51 times less effective than Gluc M_1. Gluc M_1 tightly bound to raw starch with maximum binding occurring at pH 3.0. The binding constant K of Gluc M_1 to raw starch at pH 3.0 and 4 °C was 1.6×10^5 M^{-1}. In contrast, Gluc M_2 which lacks the C-terminal region of Gluc M_1, had as small a K value as 3.2×10^3 M^{-1}, with practically no binding to the raw starch. These results indicate that only Gluc M_1 has an additional raw starch-binding site, besides the active center, in the C-terminal region.

Keywords glycoamylase; *Aspergillus saitoi*; raw starch; binding constant; raw starch-binding site; multiple form

Two glucoamylases [EC 3.2.1.3; α-D(1→4)-glucan glucohydrolase]named Gluc M_1 and Gluc M_2 were purified from *Aspergillus saitoi* and comparatively characterized.[1,2] Since these glucoamylases shared a common antigenicity and the same N-terminal amino acid sequence: H_2N·Ala–Val–Ile–Val–, it was considered that the minor glucoamylase, Gluc M_2 (molecular weight (M.W.) 70000), was an enzyme species derived from the major one, Gluc M_1 (M.W. 90000), presumably by the action of a certain proteinase(s) with removal of its C-terminal glycopeptide of about 20000 dalton (Da).

Gluc M_1 contained an active site consisting of about 7 subsites, each of which interacted with a glucose moiety of substrate,[3] as proposed by Hiromi *et al.*,[4] with a glucoamylase from *Rhizopus niveus*. Therefore, Gluc M_2 seemed to have the same active site as Gluc M_1. This supposition was confirmed in the earlier findings[1,2] that Gluc M_1 and Gluc M_2 had similar pH-activity profiles as well as specific activities in terms of unit/mol enzyme, but not of unit/mg protein, towards soluble starch as a substrate. Kinetic studies on the enzymes, however, revealed that Gluc M_2 differed from Gluc M_1 in the kinetic parameters, K_m and V_{max}, especially in the K_m values for high-molecular-weight substrates such as soluble starch and glycogen, while it differed little in both the K_m and V_{max} values for low-molecular-weight substrates such as maltose and maltotriose. The K_m values for large substrates of Gluc M_2 were consistently higher than those of Gluc M_1.[2] This indicates that only Gluc M_1, within the C-terminal glycopeptide region, has an additional site (s) that interacts with certain parts of a large substrate other than its 7 glucose moieties interacting with the active site. It is expected that the presence or absence of such an additional site (s) in a glucoamylase may especially affect the enzymatic digestion of such a high-molecular-weight substrate as raw starch which has a more complicated steric structure.

Recently we demonstrated that three forms of glucoamylase from a *Rhizopus* sp., $Gluc_1$, $Gluc_2$ and $Gluc_3$, had markedly different activities towards raw starch,[5] although having similar specific activities (unit/mol enzyme) towards soluble starch. These *Rhizopus* enzymes were in mutual relation similar to that between the *Aspergillus* enzymes, Gluc M_1 and Gluc M_2. The two minor enzymes, $Gluc_2$ (M. W. 58600) and $Gluc_3$ (M.W. 61400), were shown to be derived from the most abundant enzyme, $Gluc_1$ (M. W. 74000), by proteolysis of the N-terminal, but not the C-terminal part of $Gluc_1$.[6-8] $Gluc_1$ possessed high raw starch-binding and raw starch-digesting activities, whereas both $Gluc_2$ and $Gluc_3$ had little raw starch-binding and much lower raw starch-digesting activities.[5] We attempted, therefore, to examine the behavior towards raw starch of the two *Aspergillus* glucoamylases. The present paper deals with the different behavior of Gluc M_1 and Gluc M_2 to raw starch binding and digestion.

Materials and Methods

Chemicals Soluble starch and raw starch for use as substrates were purchased from Wako Pure Chemicals; the former was used after exhaustive dialysis against distilled water and the latter after successive washing with several changes each of distilled water and methanol, followed by drying over silica gel. The D-glucose oxidase reagent (Glucose C-Test Wako) was obtained from Wako Pure Chemicals. All other chemicals were of analytical reagent grade.

Preparation of Gluc M_1 and Gluc M_2 Gluc M_1 and Gluc M_2 were purified from a commercial digestive from *A. saitoi*, "Molsin" (Seishin Pharm. Co., Ltd.), according to the respective methods reported previously.[1,2]

Estimation of Protein Gluc M_1 and Gluc M_2 concentrations were determined from the absorbance at 280 nm by taking A_{280} (%) to be 14.97 and 14.18, respectively.[1,2]

Determination of Glucoamylase Activity For the routine assay, glucoamylase activity was determined with soluble starch as a substrate at pH 5.0 and 37 °C, according to the D-glucose oxidase method described previously.[6]

The enzymatic activity towards raw starch was determined as follows. The reaction mixture contained 20 mg of raw starch and enzyme in 1 ml of 0.1 M buffer of an appropriate pH. After incubation at 37 °C for 10 min with stirring, the reaction mixture was heated at 100°C for 1 min to terminate the reaction and then centrifuged at 3000 rpm for 10 min. The glucose liberated into the resulting supernatant was determined by the D-glucose oxidase method.

One unit of soluble starch-hydrolyzing or raw starch-digesting activity was defined as the amount of glucoamylase that liberated 1 μmol of glucose from the respective substrates per min under the specified conditions.

Estimation of Enzyme Bound to Raw Starch An enzyme solution up to 100 μl was added to raw starch up to 100 mg in 0.01 M glycine–HCl buffer (pH 3.0) containing 0.1 M NaCl to give a total volume of 1.1 ml. After incubation at 4 °C for 20 min with stirring, the suspension was transferred to a microfilter tube (Schleicher & Schuell, Inc.) with a filter paper and filtered by brief centrifugation at 4°C and 3000 rpm for a few min. The resulting filtrate was used to estimate the amount of unbound enzyme, which was determined by measuring either the enzymatic activity

on soluble starch or the absorbance at 230 nm instead of 280 nm because of the small amount of enzyme used. The amount of bound enzyme was calculated as the difference between the total and unbound enzymes, and raw starch-binding activity of enzyme was expressed as the rate (%) of binding.

Estimation of Binding Constant of Enzyme to Raw Starch The binding constant K of an enzyme-raw starch complex is expressed by Eq. 1 as follows.

$$K=\frac{B}{F(B_{max}-B)} \quad (1)$$

$$\text{thus } \frac{1}{B}=\frac{1}{B_{max}\cdot K\cdot F}+\frac{1}{B_{max}} \quad (2)$$

where B and F stand for the molar concentrations of bound and free enzymes, respectively, and B_{max} stands for the maximum molar concentration of enzyme bindable to the total amount of raw starch, which is equivalent to the total molar concentration of enzyme-binding site of raw starch. On the basis of Eq. 2, the value of K was estimated graphically from a plot of $1/B$ *versus* $1/F$.

Results

Raw Starch Digestibility of Gluc M_1 and Gluc M_2 To examine the raw starch digestibility of the two glucoamylases, raw starch (4 mg) was incubated with 4—5 units of each enzyme at 37 °C and pH 5.0 with stirring; the conditions had been used in the case of raw starch digestion with the *Rhizopus* glucoamylases[5] (Fig. 1). The digestion of raw starch with Gluc M_1 proceeded rapidly and was almost completed after a 24 h incubation. In contrast, the digestion with Gluc M_2 proceeded so slowly that a large part of the raw starch was left undigested even after such a long incubation time as 72 h (not shown here). When as large an amount as 300—400 units of Gluc M_2 was additionally added at 10 h, however, the digestion reached completion within 24 h. It appeared that Gluc M_1 and Gluc M_2 had similar degrees of digestion for raw starch of near 100%, although they had quite different rates of digestion.

pH Optima for Raw Starch Digestion by Gluc M_1 and Gluc M_2 The raw starch-digesting activity of each enzyme was determined at 37 °C as a function of pH (Fig. 2). The digestion of raw starch with the enzymes was highly pH-dependent as compared with the case of soluble starch hydrolysis; maximum digestion occurred at pH 3.5 for Gluc M_1 and pH 4.0 for Gluc M_2. The results were in contrast with the previous finding[1,2] that Gluc M_1 and Gluc M_2 had similar pH-activity curves as to soluble starch hydrolysis, with the same broad pH optima around pH 4.5.

Relative Activities towards Raw and Soluble Starches of the Enzymes The raw starch-digesting and soluble starch-hydrolyzing activities of the enzymes were determined at the respective optimal pH values and compared (Table I). It was apparent that the specific soluble starch-hydrolyzing activities in terms of unit/mg of the enzymes were in inverse relation to their molecular weights, so they showed similar values of $3.1—3.2\times10^9$ units/mol, confirming the earlier results.[1,2] On the other hand, the specific raw starch-digesting activities, not only in terms of unit/mg but also in terms of unit/mol, differed markedly between Gluc M_1 and Gluc M_2; the specific activity in unit/mol of Gluc M_2 was about 53 times lower than that of Gluc M_1. For both enzymes, the raw starch-digesting activities were lower than the soluble starch-hydrolyzing activities and the ratios of the latter to the former were 1.8 for Gluc M_1 and 91.8 for Gluc M_2. This indicates that Gluc M_2 is 51 times less effective for digestion of raw starch than Gluc M_1 when compared in terms of equiunits of soluble starch-hydrolyzing activity of each enzyme.

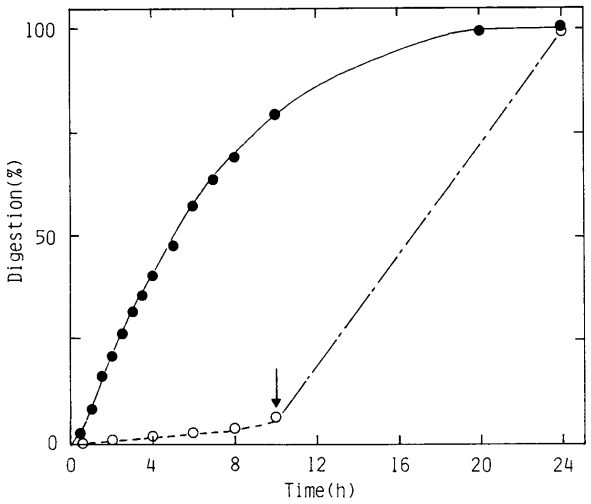

Fig. 1. Digestion of Raw Starch with Gluc M_1 and Gluc M_2

Four mg of raw starch was digested with 4—5 units of each enzyme in 3.4 ml of 0.1 M acetate buffer (pH 5.0) at 37 °C with stirring. Portions (0.25 ml) of the digestion mixtures were taken at various times with a pipette with a wide nozzle and heated at 100 °C for 1 min to terminate the reaction. The heated suspensions were centrifuged at 3000 rpm for 10 min and then the liberated glucose in the resulting supernatants was determined as described in the text. The arrow indicates the time when 350 units of Gluc M_2 was additionally added to its digestion mixture. ●, Gluc M_1; ○, Gluc M_2.

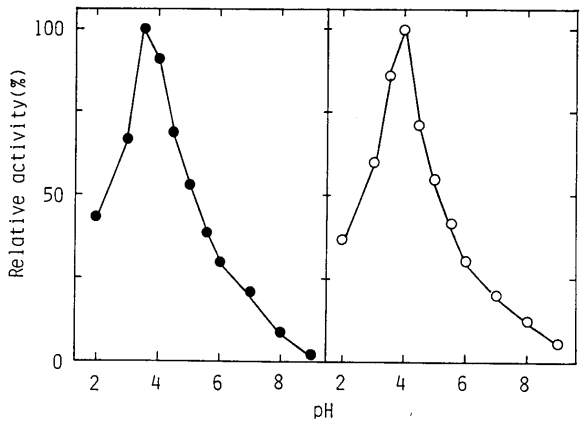

Fig. 2. Effect of pH on the Raw Starch-Digesting Activity of Gluc M_1 and Gluc M_2

The assay procedure was as described in the text. The amounts of Gluc M_1 and Gluc M_2 used were 3 and 185 units, respectively; the buffers (0.01 M, $I=0.1$) used were glycine–HCl buffer for pH 2.0—3.5, acetate buffer for pH 4.0—6.5 and borax–HCl buffer for pH 7.0—9.0. For each enzyme, the activity is expressed as a percentage of the maximum activity. ●, Gluc M_1; ○, Gluc M_2.

TABLE I. Comparison between Raw Starch-Digesting and Soluble Starch-Hydrolyzing Activities of Gluc M_1 and Gluc M_2

Enzyme	Specific activity			
	Raw starch		Soluble starch	
	(unit/mg)	(unit $\times 10^{-7}$/mol)	(unit/mg)	(unit $\times 10^{-9}$/mol)
Gluc M_1	20.0	180	35.9	3.23
Gluc M_2	0.48	3.38	44.3	3.10

Raw starch-digesting activities of Gluc M_1 and Gluc M_2 were measured at pH 3.5 and 4.0, respectively and soluble starch-hydrolyzing activities of both enzymes were measured at pH 4.5.

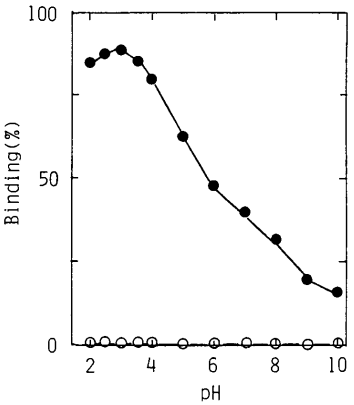

Fig. 3. Effect of pH on Binding of Gluc M_1 and Gluc M_2

Binding of the enzymes (73 μg) to raw starch (25 mg) was measured as described in the text, except that the pH was changed from 2.0—10.0. The buffers (0.01 M, $I=0.1$) used were those given in the legend to Fig. 2 and borax–NaOH buffer for pH 10.0. ●, Gluc M_1; ○, Gluc M_2.

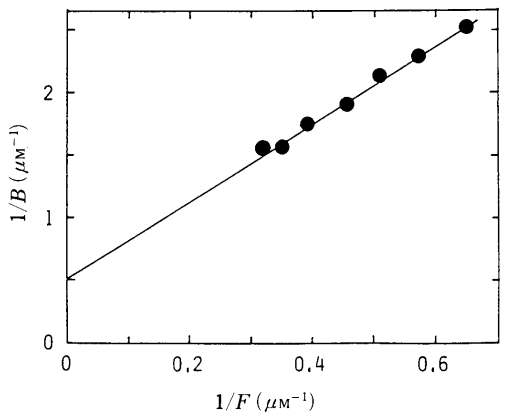

Fig. 4. Estimation of the Binding Constant of Gluc M_1 to Raw Starch at pH 3.0 and 4 °C

Binding of various amounts of Gluc M_1 (2.0—4.0 μM) to a fixed amount of raw starch (10 mg) was measured as described in the text. A plot of $1/B$ against $1/F$ was made according to Eq. 2 and least squares analysis was performed to obtain the linear slope.

Effect of pH on Raw Starch Binding of the Enzymes The effect of pH on raw starch binding of Gluc M_1 and Gluc M_2 was tested by using various buffers ($I=0.1$) of pH 2.0 to 10.0; incubation of raw starch with the enzymes was carried out at 4 °C for 20 min with stirring, the conditions being optimal for raw starch binding with $Gluc_1$.[5] As shown in Fig. 3, the binding of Gluc M_1 was considerably pH-dependent and the most favorable binding occurred at pH 3.0. However, Gluc M_2 exhibited no significant binding over the pH range tested under the same conditions as for Gluc M_1.

Binding Constants to Raw Starch of Gluc M_1 as Well as Gluc M_2 When a constant amount of Gluc M_1 (73 μg) was mixed with various amounts of raw starch (1—100 mg) at pH 3.0 and 4 °C, binding of Gluc M_1 occurred hyperbolically against the amount of raw starch, being almost complete at about 60 mg of added raw starch. To estimate the binding constant K, binding of various amounts of Gluc M_1 (390—195 μg) to a fixed amount of raw starch (10 mg) was measured at pH 3.0 and 4 °C. A plot of $1/B$ against $1/F$ was made according to Eq. 2; the linear slope, which is equal to $1/B_{max} \cdot K$, was calculated by the least squares analysis (Fig. 4). Based on the plot, the K value for Gluc M_1 was estimated to be 1.6×10^5 M^{-1}. On the other hand, since no significant binding of Gluc M_2 was again detectable under the same conditions as for Gluc M_1, it was impossible to estimate the K value for Gluc M_2, similarly to Gluc M_1. However, when a large amount of raw starch (100 mg), as compared with 10 mg for Gluc M_1, was incubated at pH 3.0 and 4 °C with a proper amount of Gluc M_2 (100 μg) so as to increase the B/F ratio, the amount of Gluc M_2 bound to raw starch became detectable although only slightly. The K value for Gluc M_2 was thus estimated to be 3.2×10^3 M^{-1}, assuming that the B_{max} value for Gluc M_2 was the same as for Gluc M_1.

Discussion

Some fungal glucoamylases have been utilized in the industrial production of glucose and ethanol from raw starch as a starting material. From the viewpoints of saving energy and simplification of the process, much attention has been paid to the enzymes capable of digesting raw starch directly without prior gelatinization. Fungal glucoamylases often exist in multiple forms varying in size and hydrolytic activity on various polysaccharides, especially on raw starch.[5,9-13] The relationship between their structures and functions is, therefore, of interest. As hererin described, two forms of *A. saitoi* glucoamylase, Gluc M_1 and Gluc M_2, the latter of which lacks the C-terminal region of the former,[2] showed marked differences in raw starch digestion and binding, as was the case with three forms of *Rhizopus* sp. glucoamylase, $Gluc_1$, $Gluc_2$ and $Gluc_3$,[5] the two latter of which lack the N-terminal region of the former.[6-8]

The optimal pH for raw starch digestion was different between Gluc M_1 (3.5) and Gluc M_2 (4.0) (Fig. 2), in spite of their having the same broad optimal pH (around 4.5) for soluble starch hydrolysis.[1,2] Such acidic but different shifts between the enzymes of the optimal pH for raw starch digestion from that for soluble starch hydrolysis may result from that Gluc M_1 bound to raw starch tightly and most favorably at pH 3.0 (Fig. 3), while Gluc M_2 scarcely bound. A similar situation as to the change in optimal pH for the two substrates was also observed with the multiple forms of *Rhizopus* sp. glucoamylase.[5]

Gluc M_1 and Gluc M_2 could digest raw starch almost completely (Fig. 1), as in the cases with soluble starch substrate.[1,2] However, they showed quite different rates of raw starch digestion in contrast to their similar rates of soluble starch hydrolysis (Table I). Gluc M_2 showed about 51 times lower rate of raw starch digestion than that of an equivalent amount of Gluc M_1 with respect to soluble starch-hydrolyzing activity. Gluc M_2 proved to be the least effective for raw starch digestion even among such *Rhizopus* enzymes having low raw starch-digesting activity as $Gluc_2$ and $Gluc_3$ as well as the two derivative enzymes from $Gluc_1$, papGluc and chymoGluc, which were found to be 27, 31, 29 and 34 times less effective, respectively, than Gluc M_1.

Low enzymatic activity towards raw starch of so-called raw starch-unadsorbable enzymes, as compared with adsorbable ones, has been shown also with the multiple forms of glucoamylases from *A. awamori* var. *kawachi*,[9-11] *A. oryzae*[12] and *R. delemar*.[13] From the results of other laboratories as well as our quantitative binding studies, it is evident that tight binding of raw starch with an enzyme

is essential for more efficient digestion of the raw starch. We first determined the binding constant K of a raw starch–glucoamylase complex for $Gluc_1$ in the previous paper[5] and for both Gluc M_1 and Gluc M_2 in the present paper. Gluc M_1 and $Gluc_1$, having high raw starch-digesting and raw starch-binding activities, had similar K values of 1.6 and $1.2 \times 10^5\ M^{-1}$, respectively. In contrast, Gluc M_2 and such *Rhizopus* enzymes as $Gluc_2$, $Gluc_3$, papGluc and chymoGluc, having low raw starch-digesting and practically little raw starch-binding activities, showed as small K values as 3.2×10^3 (Gluc M_2) and $6—8 \times 10^3\ M^{-1}$ (*Rhizopus* enzymes[14]), which are roughly 50 and 20 times smaller, respectively, than those for their original enzymes, Gluc M_1 and $Gluc_1$; Gluc M_2 having the lowest raw starch-digesting activity possessed the smallest K value, unequivocally indicating that raw starch digestion is principally dependent on the binding of raw starch with an enzyme.

It is concluded that Gluc M_1, but not Gluc M_2, possesses a separate raw starch-binding site, different from the active site, in the C-terminal region, as does $Gluc_1$ but it is in the N-terminal region. The presence of such a separate site for raw starch binding has been suggested for *E. fibuligera*[15] and *Aspergillus* sp. K-27[16] enzymes and more clearly shown with *A. awamori* var. *kawachi*,[17] *R. niveus*[18] and *R. pusillus*[19] enzymes. This conclusion is confirmed in the earlier kinetic study results[2] mentioned in the Introduction Section. Because the separate site interacts not only with raw starch but also with large soluble substrates, it would be better to designate it as a polysaccharide-binding site.

Acknowledgment The authors are greatly indebted to Dr. F. Yoshida of Seishin Pharm. Co. and Kikkoman Shoyu Co. for providing the enzyme source "Molsin".

References

1) T. Takahashi, N. Inokuchi and M. Irie, *J. Biochem.* (Tokyo), **89**, 125 (1981).
2) N. Inokuchi, T. Takahashi and M. Irie, *J. Biochem.* (Tokyo), **90**, 1055 (1981).
3) T. Koyama, N. Inokuchi, Y. Kikuchi, H. Shimada, M. Iwama, T. Takahashi and M. Irie, *Chem. Pharm. Bull.*, **32**, 757 (1984).
4) K. Hiromi, Y. Nitta, C. Numata and S. Ono, *Biochem. Biophys. Acta*, **302**, 362 (1973).
5) T. Takahashi, K. Kato, Y. Ikegami and M. Irie, *J. Biochem.* (Tokyo), **98**, 663 (1985).
6) T. Takahashi, Y. Tsuchida and M. Irie, *J. Biochem.* (Tokyo), **84**, 1183 (1978).
7) T. Takahashi, Y. Tsuchida and M. Irie, *J. Biochem.* (Tokyo), **92**, 1623 (1982).
8) T. Takahashi, Y. Tsuchida, M. Iwama, R. Ohtsuki and M. Irie, *Chem. Pharm. Bull.*, **31**, 1001 (1983).
9) S. Ueda, R. Ohba and S. Kano, *Stärke*, **26**, 374 (1974).
10) S. Hayashida, *Agric. Biol. Chem.*, **39**, 2093 (1975).
11) S. Hayashida and P. Q. Flor, *Agric. Biol. Chem.*, **45**, 2675 (1981).
12) M. N. N. Miah and S. Ueda, *Stärke*, **29**, 235 (1977).
13) J. Abe, H. Nagano and S. Hizukuri, *J. Appl. Biochem.*, **7**, 235 (1985).
14) T. Takahashi and S. Kawauchi, "unpublished data".
15) S. Ueda and B. C. Saha, *Enzyme Microb. Technol.*, **5**, 196 (1983).
16) J. Abe, K. Nakajima, H. Nagano and S. Hizukuri, *Carbohydr. Res.*, **175**, 85 (1988).
17) S. Hayashida, S. Kunisaki, M. Nakao and P. Q. Flor, *Agric. Biol. Chem.*, **46**, 83 (1982).
18) B. C. Saha and S. Ueda, *J. Ferm. Technol.*, **61**, 67 (1983); J. Abe and S. Hizukuri, *J. Jpn. Soc. Starch Sci.*, **35**, 43 (1988).
19) W. Kanlayakrit, K. Ishimatsu, M. Nakao and S. Hayashida, *J. Ferment. Technol.*, **65**, 379 (1987).