Removal of the Starch-Binding Site of *Rhizopus delemar* Glucoamylase by Limited Proteolysis with Chymotrypsin

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Rhizopus delemar glucoamylase GIII (Mw 78,000) was converted into a new form, G' (Mw 72,000), through the limited action of chymotrypsin. With the modification, its ability of adsorption on starch granules and high affinity for water-soluble polysaccharides decreased due to the removal of the postulated specific affinity site for polysaccharides, the "starch-binding site." The peptide(s), released on the modification, reduced the adsorption on raw-starch granules and the hydrolysis of glycogen with the enzymes. These results suggest that the peptide(s) is the starch-binding site(s) itself or a part of it.

From the viewpoints of saving energy and simplification of the process, there is much interest in the direct saccharification of raw starches in ethanol production and other fermentation industries. Some fungi produce several forms of glucoamylases (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) which differ in raw starch digesting-activity, and the relationship between their structures and functions is of interest. In a previous paper,¹⁾ we reported the purification of three forms (a raw-starch digesting form, GIII, and two non-digesting forms, GI and GII) of glucoamylase from *Rhizopus delemar*, and concluded that GI and GII were derived from GIII through the limited action of the protease (possibly in cooperation with glycosidase) in the crude enzyme preparation, and that only GIII had a specific affinity site, a starch-binding site, for polysaccharides.

Here, we attempted to modify *R. delemar* glucoamylase GIII with chymotrypsin to determine whether or not a similar modification to the post-secretional modification of the native enzyme into the site-less form occurs, and investigated the properties of the resulting modified enzyme and peptide(s).

MATERIALS AND METHODS

Enzymes. *Rhizopus delemar* glucoamylases GI, GII and GIII were purified as described previously.¹⁾ Chymotrypsin (Type II) was a product of Sigma.

Materials. Maltooligosaccharides were donated by Hayashibara Biochemical Laboratory. Glycogen (rabbit liver), prepared by the method of Carrol *et al.*,²⁾ was generous gift from Dr. M. Nakagawa, Kagoshima University. Potato amylopectin was fractionated in our laboratory by the method of Lansky *et al.*³⁾ under a nitrogen atmosphere. Other reagents were of the highest grade commercially available.

Methods. Glucoamylase activity was determined as follows: A reaction mixture of 200 μ l of a 2% soluble starch solution and 50 μ l of the enzyme solution in 50 mM sodium acetate buffer (pH 4.5) containing 0.025% bovine serum albumin was incubated at 40°C for 15 min. Then the reaction was stopped by immersing the tube in a boiling water bath for 30 sec. The resulting glucose was determined by the glucose oxidase-peroxidase method.⁴⁾

One unit is defined as the amount of

enzyme which hydrolyzes $1\ \mu\text{mol}$ of α -1,4-glucosidic bond per min under the conditions used.

Protease activity was measured as follows: A reaction mixture of 1% casein in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM CaCl_2 and $20\ \mu\text{l}$ of the enzyme solution was incubated at 37°C for 30 min. Then the reaction was stopped by an addition of 1 ml of 0.4 M trichloroacetic acid (previously chilled in an ice bath), and then the supernatant obtained on centrifugation was retained. The activity was expressed as the absorbance at 280 nm of the supernatant.

Protein was determined by the method of Lowry *et al.*⁵⁾ and from the absorbance at 280 nm. Total carbohydrate was determined by the method of Koehler.⁶⁾

Fig. 1. Changes in the disc-gel (A) and SDS-gel (B) electrophoresis patterns for glucoamylase during limited proteolysis.

The reaction mixture was electrophoresed in 5% (A) and 7.5% (B) polyacrylamide gels. The proteins were stained with Coomassie brilliant blue R-250. A, B, C, D and E, incubation for 0 hr, 3 hr, 6 hr, 9 hr and 24 hr, respectively; F, incubation for 24 hr without addition of chymotrypsin.

RESULTS AND DISCUSSION

Limited proteolysis with chymotrypsin

Rhizopus delemar glucoamylase GIII (Mw 78,000) was degraded partially with chymotrypsin, being converted into a new form, G' (Mw 72,000, Fig. 1). The activity (U/ml) toward soluble starch remained constant throughout the proteolysis.

Glucoamylase G', the released peptide(s) and chymotrypsin were separated from each other by chromatography on a column of Bio-Gel P-30 (Fig. 2). Glucoamylase and protease activities were found in fr. 19~40 and fr. 50~80, respectively. Glucoamylase G' (fr. 19~24) gave a single band on disc-gel and SDS-gel electrophoresis. No enzymatic activity was found in fr. 83~120, which were pooled as the peptide fragment fraction.

Properties and actions of G'

The glucoamylase G' showed the same temperature stability (up to 45°C at pH 4.5 for 30 min) as that of the native enzyme. The kinetic constants for water-soluble substrates of the native and modified enzymes are compared in Table 1. It is obvious that: 1) G' shows almost the same K_m and slightly larger V_{max} values for oligosaccharides than the native enzyme; 2) the K_m and V_{max} values of G' for polysaccharides are 30~300 and 1.1~1.5 fold, re-

Fig. 2. Chromatography of a reaction mixture after proteolysis on Bio-Gel P-30.

The column size was $2.6 \times 95\text{ cm}$ and 5 ml fractions were collected at a flow rate of 19 ml/hr. —●—, Glucoamylase; ---▲---, chymotrypsin; —○—, protein (A_{280}).

Table 1. Kinetic constants of *Rhizopus delemar* glucoamylases.

	G'		GIII ^{a)}		GII ^{a)}	
	$K_m^{b)}$ (mM)	V_{max} (U/nmol)	$K_m^{b)}$ (mM)	V_{max} (U/nmol)	$K_m^{b)}$ (mM)	V_{max} (U/nmol)
Maltose	1.8	0.69	2.0	0.61	1.9	0.84
Maltotriose	0.56	3.2	0.64	2.6	0.57	3.0
Maltotetraose	0.26	7.1	0.26	5.8	0.21	7.5
Maltopentaose	0.16	7.1	0.17	5.7	0.14	7.2
Maltohexaose	0.15	8.6	0.16	6.0	0.11	8.8
Maltoheptaose	0.14	8.0	0.15	5.5	0.10	8.1
Glycogen	0.24	4.5	0.0008	3.9	0.30	4.8
Amylopectin	0.01	5.1	0.0003	4.8	0.007	5.2

^{a)} Cited from Ref. 1.

^{b)} On the basis of the non-reducing, terminal residues.

Fig. 3. Actions of the native (●) and modified (▲) glucoamylase on raw starch.

(A) Adsorption on raw starch: various amounts of raw wheat starch and 10U of the enzymes were incubated at 4°C for 20 min. The adsorption as a percentage was calculated from the residual activity in the supernatant.

(B) Hydrolysis of raw starch: the reaction mixtures, containing 250 mg (dry weight basis) of starch and 25 U of the enzymes in 5 ml of 50 mM sodium acetate buffer (pH 4.5), were incubated at 40°C under mild stirring.

spectively, those in the case of GIII. Thus, the proteolytic action decreased the affinity for high-molecular weight substrates such as glycogen and amylopectin without affecting that for oligosaccharides. Almost all the constants are similar to the respective values for *R. delemar* glucoamylase GII, which has been suggested to be derived from GIII through partial proteolysis with the protease in the crude enzyme preparation.¹⁾ Takahashi *et al.*⁷⁾ also reported that *Rhizopus* sp. glucoamylase decreased the affinity for polysaccharides by

Fig. 4. Chromatography of the released peptide fraction on Toyopearl HW-40S.

The column size was 2.6 × 95 cm and 5 ml fractions were collected at a flow rate of 19 ml/hr.

the limited proteolysis with chymotrypsin and papain. However, the increases of the K_m values of the modified glucoamylases for glycogen and amylopectin were only 5 and 9 folds, respectively, with the considerable decreases of the molecular weight to 87 and 77% of its original form (Mw 73,600) by the modification, respectively, with chymotrypsin and papain.

The abilities of adsorption on and digestion of starch granules were shown to decrease to $\sim 1/9$ and $\sim 1/4$, respectively, with the limited proteolysis (Fig. 3, A and B).

The results mentioned above imply that the affinity for high-molecular weight substrates, both water-soluble and -insoluble ones, was decreased by the limited proteolysis, probably

Fig. 5. Effect of the fragment(s) on the catalytic and adsorbing actions of glucoamylases.

(A) Adsorption on raw starch: for conditions, see Fig. 3.

(B) Hydrolysis of glycogen: the reaction mixture contained 0.25% rabbit liver glycogen and 0.05 U of enzyme with or without 200 μ g of the large-molecular fragment(s) in 2 ml of 50 mM sodium acetate buffer (pH 4.5) at 40°C.

●, GIII; ▲, G'; ○, GIII + fragment(s); △, G' + fragment(s).

due to removal of the starch-binding site without any damages in the catalytic site.

Properties of the peptide fragment

The fragment fraction (fr. 83~120 in Fig. 2) is a mixture of fragments of various sizes, as found on gel-chromatography (Fig. 4). The main large-molecular fraction (fr. 75~93), which may also be a mixture, was pooled and concentrated for further studies. The fragment(s) (200 μ g) causes 41 and 24% decreases in the adsorption of *R. delemar* glucoamylase GIII (10 U) on 80 and 200 mg of raw wheat starch, respectively (Fig. 5A), and 15% of the fragment(s) was adsorbed on 200 mg of granules. These results suggested that the fragment(s) has an affinity for starch granules and thus inhibits adsorption of the enzyme on the substrate. It inhibited the hydrolysis of glycogen with both the native and the modified enzyme also (Fig. 5B). Namely, 19 and 48% decreases in the hydrolysis with GIII and G', respectively, were observed on 9 hr incubation with the addition of the fragment(s) to the reaction mixture. The greater inhibition of modified glucoamylase G' than of the native glucoamylase by the fragment(s) would be due to the lower affinity of glucoamylase G' for polysaccharides.

In conclusion, these results provided the supporting evidence for the presence of the "starch-binding site" in glucoamylase GIII, and the fragment(s) obtained on the proteolysis is the starch-binding site itself or its main part. Hayashida *et al.* reported⁹⁾ that an affinity site for raw starch existed in the glucoamylase of *Aspergillus awamori* var. *kawachi*, and that the site could be removed through proteolysis with subtilisin. Saha and Ueda⁹⁾ and Takahashi *et al.*¹⁰⁾ obtained glycopeptides from crude preparations of the glucoamylases of *Aspergillus niger* and *Rhizopus* sp., and found that these glycopeptides inhibited the action of glucoamylase on raw starch. Therefore, it is worthwhile paying attention to the proteolytic activity, which possibly converts the glucoamylase into a less active form as to raw starch, in the culture medium for the production of raw-starch digesting glucoamylases.

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キモトリプシンによる *Rhizopus delemar*
グルコアミラーゼの限定分解

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Rhizopus delemar グルコアミラーゼ GIII (Mw 78,000) をキモトリプシンで限定分解し, 修飾酵素 G' (Mw 72,000) とペプチドを得た. G' は, 可溶性澱粉溶液に対しては GIII と同じ活性を示したが, 生澱粉への吸着力および分解活性は, それぞれ GIII の約 1/9 と

1/4 であった. また, G' は, 直鎖のオリゴ糖に対しては GIII とほとんど同じ K_m 値を示したが, アミロペクチンやグリコーゲンに対しては 30~300 倍の大きな値を示し, キモトリプシン処理により, 明らかに高分子基質に対する親和性が減少した. これらの事実は, GIII には, 活性部位とは別の「澱粉結合部位」が存在することを支持し, 限定分解によりこの部位が除かれて G' が生成することを強く示唆する. 限定分解により得られたペプチド中の最も高分子量のものは, 生澱粉に吸着性を有し, GIII や G' のグリコーゲンの分解や生澱粉への吸着を阻害した. これらの結果は, このペプチドが澱粉結合部位そのもの, あるいはその一部であることを示唆する.