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PATTERNS OF ACTION OF GLUCOAMYLASE ISOZYMES FROM *Aspergillus* SPECIES ON GLYCOGEN

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ABSTRACT

Glucoamylase isozymes from black *Aspergillus* species have been freed of all traces of *alpha*-amylase by chromatography on Bio-Gel P-100, as evidenced by limited hydrolysis of oxidized amylose. Glucoamylase I retains its ability to hydrolyze rabbit-liver glycogen rapidly. By contrast, glucoamylase II hydrolyzes glycogen slowly, and addition of *alpha*-amylase to glucoamylase II does not enhance its activity toward glycogen. These results indicate that *alpha*-amylase is not involved in hydrolysis of glycogen by glucoamylase.

INTRODUCTION

Glucoamylase (EC 3.2.1.3) produced by strains of black *Aspergillus* was shown by Pazur and Ando[1], Smiley and Hensley[2], and Watanabe and Fukimbara[3] to be a mixture of two enzymes having very similar properties. Smiley *et al.*[4] demonstrated that the two forms of the enzyme, designated glucoamylase I and glucoamylase II, differ in their kinetics of action on glycogen. Glucoamylase I readily attacks glycogen, whereas glucoamylase II attacks it very slowly. Marshall and Whelan[5] recently showed that glucoamylases from *Aspergillus niger* and *Rhizopus niveus* differ in their action on partially oxidized amylose. The *R. niveus* enzyme releases D-glucose from nonreducing ends of the oxidized amylose chains until further action is blocked, presumably at an oxidized D-glucose residue. *Aspergillus niger* glucoamylase, however, did not appear to be blocked by oxidized residues in the amylose chain; this was attributed to contamination of the glucoamylase with *alpha*-amylase, which ruptures internal D-glucosidic bonds of the oxidized amylose, and produces new, unmodified end-groups for glucoamylase attack[5]. Contamination of glucoamylase I (from *A.*

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niger) with *alpha*-amylase could explain the facility with which it hydrolyzes glycogen. The work reported in this paper shows that (*a*) contamination of *A. niger* glucoamylase with *alpha*-amylase cannot explain the different patterns of the action of isozymes on glycogen, and (*b*) glucoamylase I as normally isolated from columns of DEAE-cellulose contains minute proportions of *alpha*-amylase, but that the glucoamylase II so isolated is free from *alpha*-amylase.

MATERIALS AND METHODS

Source of glucoamylase. — Glucoamylase was prepared by the method of Smiley *et al.*[6] from *A. niger* NRRL 337, *A. cinnamomeus* NRRL-5285, and *A. awamori* NRRL-3112. A commercial sample of crude glucoamylase (Diazyme) was obtained from Miles Chemical Co. Crystalline glucoamylase from *Rhizopus niveus* was a product of Miles Laboratories, Inc., Kankakee, Illinois.

Source of carbohydrates. — Lintner starch and rabbit-liver glycogen were products of Pfanstiehl Chemical Co. Potato amylose, mol. wt. 800,000, was prepared in this laboratory, and was kindly supplied by Mr. Robert Tobin. Oxidized amylose was prepared by the method of Marshall and Whelan[5]. Unbranched malto-oligosaccharides of d.p. 3 to 10 were prepared at NRRL by Mr. Henry Zobel.

Chromatographic supports. — Whatman DE-52 DEAE-cellulose was purchased from Schleicher & Schull Co., N.J.; Sephadex G-100 and Blue Dextran 2000, from Pharmacia Co., N.J.; and Bio-Gel P-100, from Bio Rad Corp., Calif.

Preparation of isozymes. — The glucoamylase isozymes were prepared essentially as described previously[4], except that the DEAE-cellulose was equilibrated with 0.05M acetate buffer at pH 4.2, and eluted with a linear gradient of acetate buffer (0.05 to 0.5M) at the same pH. Under these conditions, *alpha*-amylase, if present, appeared between the two glucoamylase isozymes.

Gel-permeation chromatography. — A column (1.0 × 200 cm) of Bio-Gel P-100 (100–200 mesh) was equilibrated with 0.1M acetate buffer, pH 4.2. Amylase samples containing 3–5 mg of protein were placed on the column, and eluted with the same buffer at a flow rate of ~7.5 ml/h, with collection of 2.5-ml fractions. Blue dextran, serum albumin, and ovalbumin were used as marker substances.

For comparative purposes, similar amylase preparations were chromatographed on a column (1.2 × 90 cm) of Sephadex G-100 prepared as just described, and samples were eluted at a flow rate of 10.0 ml/h.

Paper chromatography. — Starch hydrolysis-products were chromatographed on Whatman No. 1 paper, with 6:4:3 butanol–pyridine–water as the irrigant. The sugars were made visible by a dip reagent[7] of alkaline silver nitrate.

Assay of amylolytic activity. — Glucoamylase activity was measured according to the method described by Miles Chemical Co.[8], as modified by Smiley *et al.*[4]. A unit of activity is defined as the amount of enzyme needed in order to liberate 1 g of D-glucose from 4% soluble starch in 1 h at 60°. For convenience, *alpha*-amylase activity is also expressed as grams of D-glucose-equivalent released under the condi-

tions just described. True D-glucose was measured by the D-glucose oxidase method of Hill and Kessler[9].

Enzymic digestion of oxidized amylose. — Oxidized amylose (100 mg) was dissolved in 7.5 ml of 0.1M acetate buffer, pH 5.0. Enough enzyme was added to provide 0.1 to 0.2 unit, and the volume was made to 10 ml with buffer. Incubation was conducted at 37°, and samples were taken at suitable time-intervals to be assayed for reducing sugar and true D-glucose.

Amylolytic reaction rate for glycogen. — The initial velocities of the amylolytic reactions on glycogen were measured with a Technicon Auto Analyzer as previously described[4].

Disc gel-electrophoresis. — Disc electrophoresis in poly(acrylamide) was performed by Nerenberg's procedure[10] by using a Tris–diethylbarbituric acid buffer (1.0 g/liter and 5.52 g/liter, respectively) at pH 7.4.

Zone electrophoresis. — Zone electrophoresis was performed in Gelman electrophoresis equipment, by using strips of cellulose acetate (Sepraphore III) and Gelman High Resolution Buffer, pH 8.8, I = 0.06. The bands were stained either with periodic acid–Schiff reagent or Coomassie Brilliant Blue dye.

Acid stability. — The pH values of the amylolytic enzymes were adjusted with hydrochloric acid to the desired pH, and the solutions held for various periods at 37°. At the end of the holding period, the pH was adjusted to 4.2 with 10M sodium hydroxide, and the residual activity was measured.

RESULTS

DEAE-cellulose chromatography. — Culture filtrates from *A. niger* NRRL-337 and *A. cinnamomeus* NRRL-5285 gave three amylolytic peaks when chromatographed on DEAE-cellulose (see Figs. 1A and 1B). In each case, the first and third peaks consisted of a glucoamylase, as evidenced by the 1:1 ratio between the reducing sugars as assayed by the alkaline ferricyanide procedure and the true D-glucose as measured by D-glucose oxidase. The second peak consisted of *alpha*-amylase, as evidenced by the low ratio of true D-glucose formed to the total reducing sugars. Further confirmation as to the type of amylase in each peak was obtained by paper chromatography of its starch hydrolysis-products. For both organisms, the first and third peaks contained only D-glucose, and the second peak gave several malto-oligosaccharides, ranging from maltose to maltohexaose, with only traces of D-glucose. Two peaks of glucoamylase activity were obtained when Diazyme extracts were chromatographed on DEAE-cellulose (see Fig. 1C). (Diazyme is prepared by use of a high-glucoamylase-producing strain of a black *Aspergillus*, and the manufacturer claims that the product contains only traces of *alpha*-amylase.) Under the chromatographic conditions described, a small amount of *alpha*-amylase would probably not be separated from the large glucoamylase peaks. *Aspergillus awamori* NRRL-3112 also gave only two peaks of amylolytic activity, and resembled Diazyme in this respect.

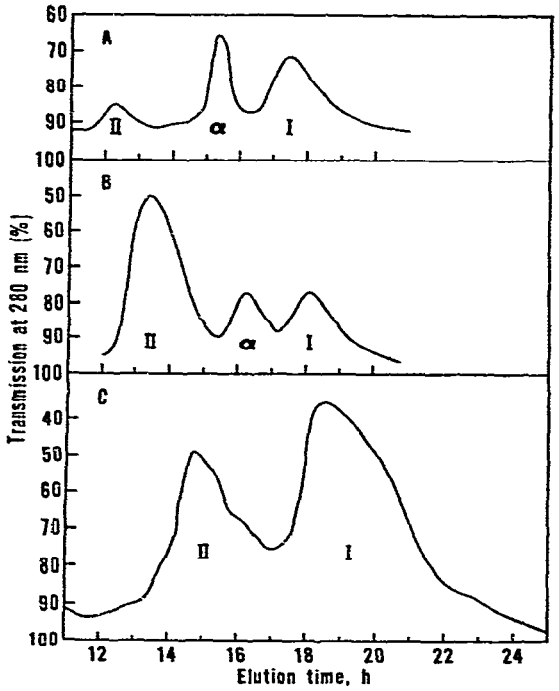

Fig. 1. Elution patterns of *Aspergillus* amylases from columns of DEAE-cellulose. [(A) *Aspergillus niger* NRRL 337, (B) *A. cinnamomeus* NRRL 5285, (C) Diazyme. I and II refer to glucoamylase I and II, respectively. The symbol α designates the *alpha*-amylase peak.]

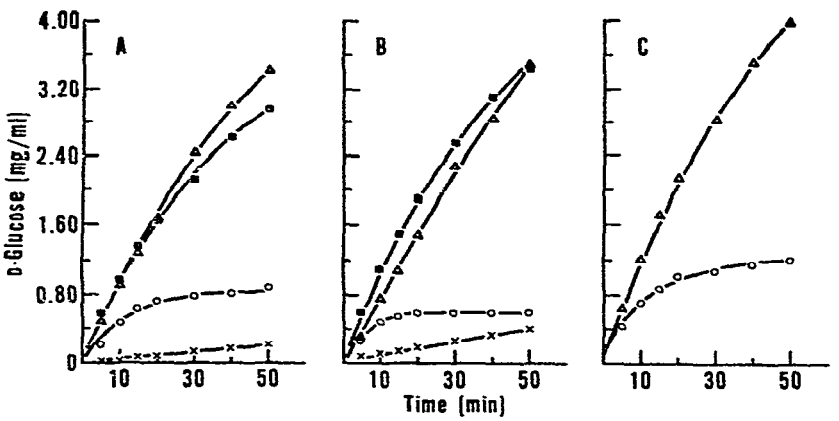

Fig. 2. Patterns of action of *Aspergillus* amylases on partially oxidized amylose. [(A) *Aspergillus niger* NRRL 337, (B) *A. cinnamomeus* NRRL 5285, (C) Diazyme. Glucoamylase I, Δ– – –Δ; glucoamylase II, O———O; *alpha*-amylase, ×———×; glucoamylase II+*alpha*-amylase, ■———■. D-Glucose determined by D-glucose oxidase.]

Reaction of glucoamylase isozymes and alpha-*amylase on oxidized amylose and glycogen.* — The three amylolytic peaks isolated from *A. niger* NRRL-337 and *A. cinnamomeus* NRRL-5285, and the two isolated from a Diazyme extract, were tested for their action on partially oxidized amylose by the method of Marshall and Whelan[5]. The results are illustrated in Fig. 2. Glucoamylase II liberates only limited amounts of free D-glucose from the substrate, indicating that the hydrolytic action is blocked at the oxidized sites in the amylose chain. Glucoamylase I continues to release D-glucose from oxidized amylose during the course of the experiment, suggesting an active, endo-amylase activity which could be provided by a trace of *alpha*-amylase in these preparations. Addition of *alpha*-amylase to glucoamylase II results in D-glucose production comparable to that obtained with glucoamylase I. These results would be predicted, based on the work of Marshall and Whelan[5].

Addition of *alpha-amylase* to glucoamylase II would be expected to cause the initial rate of glycogen degradation catalyzed by the latter enzyme to resemble the action of glucoamylase I. Accordingly, the kinetic experiments described in ref. 4 were repeated, using mixtures of *alpha*-amylase and glucoamylase II. Table I lists the results. Glucoamylase II obtained either from *A. niger* or *A. cinnamomeus* converts

TABLE I

INITIAL RATE OF FORMATION OF REDUCING SUGAR FROM GLYCOGEN BY AMYLASES OF *Aspergillus cinnamomeus* NRRL 5285 AND *A. niger* NRRL 337

Glycogen (mg/ml)	*Apparent* D-*glucose (mg/ml/min)*							
	Glucoamylase I (0.005 units)		*Glucoamylase II (0.005 units)*		*α-Amylase (0.005 units)*		*α-Amylase (0.0025 units)+ glucoamylase II (0.0025 units)*	
	NRRL 5285	*NRRL 337*	*NRRL 5285*	*NRRL 337*	*NRRL 5285*	*NRRL 337*	*NRRL 5285*	*NRRL 337*
1	0.68	0.62	0.06	0.10	0.04	0.06	0.04	0.08
2	1.21	1.08	0.14	0.13	0.06	0.10	0.07	0.12
3	1.64	1.55	0.21	0.18	0.09	0.12	0.12	0.15
4	2.09	2.04	0.29	0.20	0.10	0.15	0.16	0.20
5	2.61	2.46	0.39	0.22	0.12	0.18	0.21	0.24

less than 10% of the glycogen into D-glucose at all substrate concentrations tested. Glucoamylase I converts well over half of the glycogen into D-glucose under the same conditions. In contrast to the results obtained with oxidized amylose, addition of *alpha*-amylase from these organisms to glucoamylase II does not alter the production of D-glucose from glycogen; the reason it does not enhance formation of D-glucose is the inability of these *alpha*-amylases to attack glycogen readily. Therefore, the facility with which glucoamylase I attacks glycogen cannot be due to contamination by *alpha*-amylase.

The effect of *alpha*-amylase on the hydrolysis of glycogen by glucoamylase II from Diazyme was also tested. As *alpha*-amylase could not be isolated from Diazyme, *alpha*-amylases from *A. niger* and *A. cinnamomeus* were used. The results in Table II demonstrate that, if the level of glucoamylase II is kept constant and increasing levels

TABLE II

NON-EFFECT OF *alpha*-AMYLASE OF *Aspergillus niger* 337 ON PRODUCTION OF D-GLUCOSE FROM GLYCOGEN BY GLUCOAMYLASE II[a]

α-Amylase (μg)	*Glucose (mg/ml)*
0	0.54
10	0.50
20	0.54
40	0.57
80	0.63

[a]Incubation of 0.075 unit/ml (equivalent to 60 μg of protein) for 6 min at 60°.

of *alpha*-amylase are added, no significant increase in the production of D-glucose is observed. These results also indicate that *alpha*-amylase is without effect on the glucoamylase II activity from Diazyme.

Gel filtration. — Glucoamylase I could be completely separated from contaminating *alpha*-amylase on Bio-Gel P-100 columns. It was not possible to effect complete separation of these two enzymes by use of Sephadex G-100, even though the elution volumes were quite different (see Table III). The order of elution of *alpha*-amylase in respect to glucoamylase I is reversed on the two gels. *alpha*-Amylase

TABLE III

CHROMATOGRAPHY OF *Aspergillus cinnamomeus* AMYLASE ON SEPHADEX G-100 AND BIO-GEL P-100

Amylase chromatographed	*Ve/Vo*[a]	
	Sephadex G-100	*Bio-Gel P-100*
Glucoamylase I	1.98	1.44
alpha-Amylase	1.59	1.72

[a]Ve = elution volume of sample; Vo = void volume of column (*e.g.*, elution volume of Blue Dextran).

elutes after glucoamylase I on Bio-Gel P-100, and this behavior precludes contamination of glucoamylase I by "tailings" from the *alpha*-amylase peak. Glucoamylase I from the Bio-Gel column was shown to be free from *alpha*-amylase, as it failed to hydrolyze oxidized amylose significantly (see Fig. 3A). Glucoamylase I from the column of Sephadex G-100 readily hydrolyzes oxidized amylose; this indicates that

it is still contaminated with *alpha*-amylase[5] (see Fig. 3A). Glucoamylase I from the Bio-Gel column was tested against glycogen; the activity proved to be identical to that found before chromatography on Bio-Gel (see Fig. 3B). It therefore seems quite definite that glucoamylase I acts independently of *alpha*-amylase on glycogen.

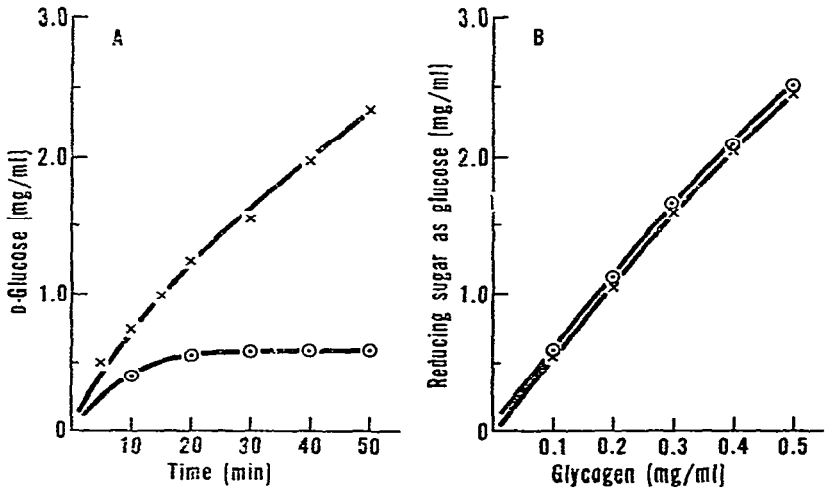

Fig. 3. Comparison of action patterns of glucoamylase I fractions, obtained by gel-permeation chromatography, on (3A) oxidized amylose, (3B) rabbit-liver glycogen. [Sephadex G-100, ×———×; Bio-Gel P-100, ⊙———⊙. Organism, *A. cinnamomeus* NRRL 5285.]

Inversion in the elution order of *alpha*-amylase and glucoamylase I from the two gels may be due to their difference in absorption on the gels; this could negate an elution order based strictly on molecular size.

Electrophoresis. — It should be mentioned that no *alpha*-amylase could be detected in glucoamylase I fractions from DEAE-cellulose by either zone or disc-gel electrophoresis. The migration distances of the two enzymes are sufficiently different that it should be possible to separate them. Apparently, the concentration of contaminating *alpha*-amylase is too low to be detected by these techniques. Electrophoresis is not a suitable tool for measuring the purity of glucoamylase I preparations.

Acid stability. — Phillips and Caldwell[11] were able to destroy the *alpha*-amylase in *R. delemar* glucoamylase preparations completely by holding the enzyme at pH 2.3 for 9 days at 8°. Fractions of glucoamylase I, from *A. niger* and *A. cinnamomeus*, obtained by chromatography on a column of DEAE-cellulose, were not altered by this procedure. By lowering the pH to 2.0, it could be demonstrated that *alpha*-amylase is more labile than glucoamylase I (see Fig. 4). Complete inactivation of the *alpha*-amylase did not occur, as the treated enzyme solution actively hydrolyzed oxidized amylose (see Fig. 5); this was confirmed on mixing *alpha*-amylase with glucoamylase II free from *alpha*-amylase and treating at pH 2.0 in the same way: the treated mixture readily hydrolyzed oxidized amylose. It is not yet known why

alpha-amylase is not destroyed by this treatment; possibly, glucoamylase protein protects the *alpha*-amylase under these conditions.

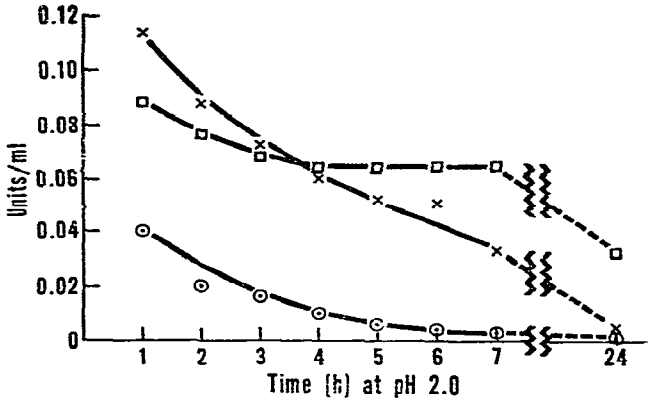

Fig. 4. Inactivation of amylases of *A. niger* NRRL 337 at pH 2.0. [Glucoamylase I, ×———×; glucoamylase II, □———□; *alpha*-amylase, ⊙———⊙.]

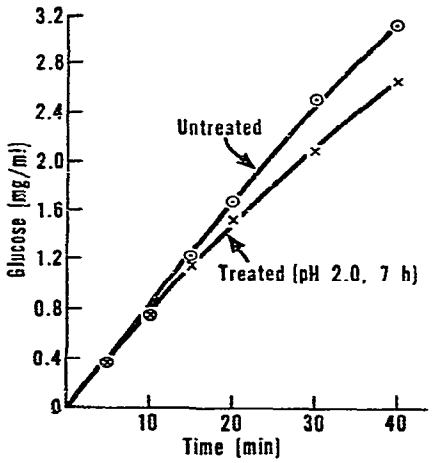

Fig. 5. Lack of effect on action pattern on partially oxidized amylose by treatment of *A. niger* NRRL 337 at pH 2.0. [Untreated control, ⊙———⊙; treated at pH 2.0 for 7 h, ×———×.]

DISCUSSION

Both glucoamylase isozymes have now been prepared free from *alpha*-amylase, as indicated by their inability to hydrolyze oxidized amylose completely. The *alpha*-amylase-free isozymes still differ in their action on rabbit-liver glycogen. Glucoamylase I readily hydrolyzes glycogen, whereas glucoamylase II shows very little action thereon. The highly purified glucoamylase I also resembles *R. niveus* glucoamylase,

which readily hydrolyzes glycogen, but cannot completely degrade oxidized amylose[5] (see Fig. 6).

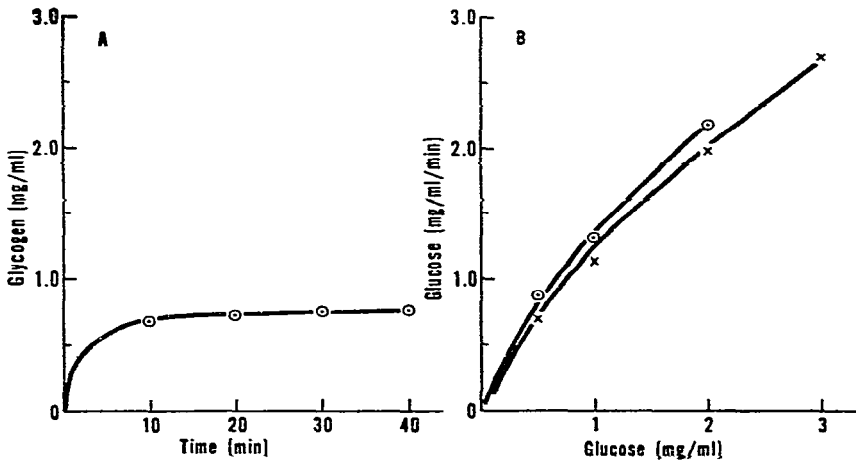

Fig. 6. Action of *Rhizopus niveus* glucoamylase on oxidized amylose and glycogen. [(A) Oxidized amylose, (B) glycogen (initial velocity). *R. niveus*, ⊙——⊙; glucoamylase I (Diazyme) control, ×——×.]

The difference in rate of hydrolysis of substrate by the two isozymes may be due to difference in the degree of branching. It has been shown[4] that glucoamylase I attacks both soluble starch and waxy-maize amylopectin faster than does glucoamylase II, although the difference is not so great as with glycogen that is more highly branched. There are no differences in rates when the substrates are unbranched malto-oligosaccharides of d.p. 2 to 10. It is, therefore, conceivable that glucoamylase II cannot hydrolyze α-D-(1→6) branch-points as readily as does glucoamylase I. It should, however, be noted that rabbit-liver glycogen was the only glycogen source tested. It is possible that the *alpha*-amylase-free glucoamylase I herein described would not act so readily on glycogen from other sources (such as cat liver) and that it might resemble *R. niveus* glucoamylase in this respect[12].

The results of this study indicate that the difference in action of glucoamylases I and II on glycogen cannot be ascribed to contaminating traces of *alpha*-amylase. Thus, glucoamylase I acts on glycogen and starch as a pure exo-D-glucosidase. The enzyme proceeds to release D-glucose from the nonreducing end of a branch as an α-D-(1→4)-glucosidase until it reaches the branching point, whereupon it exhibits α-D-(1→6)-glucosidase activity. In this way, glucoamylase I catalyzes complete hydrolysis of the polysaccharide, releasing only D-glucose in the process.

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