Note

Spectrophotometric Assay for Amylo-1,6-Glucosidase Using 6-*O*- α -D-Glucosyl Cyclomaltoheptaose (Glucosyl β -Cyclodextrin)

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A spectrophotometric assay procedure for amylo-1,6-glucosidase (EC. 3. 2. 1. 33) using 6-*O*- α -D-glucosyl cyclomaltoheptaose (glucosyl β -cyclodextrin) as a substrate was described in detail. The enzyme showed optimum activity around pH 6.3 and a K_m of 8.3 mM for glucosyl β -cyclodextrin, and produced β -D-glucose. The results of this assay well agreed with those of the assay involving the reverse reaction using [^{14} C] glucose as a substrate.

Amylo-1,6-glucosidase (EC. 3. 2. 1. 33) is very useful for the structural analysis of branched maltosaccharides since the enzyme cleaves a glucose unit in α -1,6 linkage.¹⁾ The purification procedure of the enzyme from rabbit muscle was reported,²⁾ but the determination of the activity was not so easy. The reverse reaction assay³⁾ requires the use of radioactive material and expensive facilities. An alternative glucose production assay was not examined in detail because the substrates of 6³- α -D-glucosyl maltotetraose⁴⁾ and 6-*O*- α -D-glucosyl cyclomaltooligosaccharides (glucosyl cyclodextrins)⁵⁾ were not easily available. Recently, an easy preparative method for glucosyl cyclodextrins was developed. The method involves the synthesis of branched cyclodextrins through the condensation of cyclodextrins and maltose or maltotriose with isoamylase,⁶⁾ and the trimming of the side chains with glucoamylase.⁷⁾ We report here a convenient spectrophotometric assay procedure for amylo-1,6-glucosidase using 6-*O*- α -D-glucosyl cyclomaltoheptaose (glucosyl β -cyclodextrin) as the substrate.

The assay procedure comprised the hydrolysis of glucosyl β -cyclodextrin, which is a better substrate than 6-*O*- α -D-glucosyl cyclomaltohexaose (glucosyl α -cyclodextrin),⁸⁾ followed by determination of the released glucose with

glucose oxidase and peroxidase. The purified rabbit muscle enzyme yielded the theoretical amount of glucose from glucosyl β -cyclodextrin,⁷⁾ and produced β -D-glucose (Fig. 1) which was the opposite glucose anomer produced from phosphorylase limit dextrin.⁹⁾ The pH-dependent activity profile and the pH for the optimum activity were similar to those in the case of the reverse reaction assay³⁾ (Fig. 2). The optimum pH was slightly higher than those (pH 5.5-5.9) reported previously.^{5,8)} Figure 3 shows the dependence of the activity on the concentration of glucosyl β -cyclodextrin, and that the K_m value was 8.3 mM. Based on these results, we chose pH 6.3 and 40 mM glucosyl β -cyclodextrin as the standard assay conditions. Under these conditions, the activity was proportional to the amount of enzyme, up to the release of 45 nmol of glucose, and to the incubation time, up to 1 hr. 2-Mercaptoethanol in the enzyme solution inhibited the glucose oxidase-peroxidase reaction but this was overcome by the preincubation with N-ethylmaleimide. Table 1 shows the glucose-production and glucose-incorporation activities of the crude, partially purified and purified enzyme preparations from rabbit muscle. All the preparations showed the same ratio for the two activities, indicating that the results of our spectrophotometric assay well agree with

Fig. 1. Anomer analysis of D-glucose produced from glucosyl β -cyclodextrin with amylo-1,6-glucosidase.

Glucosyl β -cyclodextrin (40 mM) was incubated at 37°C with the enzyme (0.56 U/ml) at pH 6.4. After 1 min the reaction was terminated by the addition of a mixture of chloroform and methanol. The supernatant obtained by centrifugation was incubated with 2,2'-azino-di-(3-ethylbenzthiazoline)-6-sulphonate (ABTS), peroxidase and glucose oxidase, and the absorbance (405 nm) of oxidized ABTS produced on oxidation of β -D-glucose was determined. The arrow indicates the time of addition of mutarotase.

those of the assay involving the reverse reaction.³⁾

EXPERIMENTAL

Materials. Rabbits (male, 2 kg) were purchased from Kyudo Co., Ltd. Glucosyl β -cyclodextrin was prepared as described previously.^{6,7)} ω -Aminoethyl Sepharose 4B was prepared by the method of Shaltiel.¹⁰⁾

Preparation of amylo-1,6-glucosidase. The enzyme was prepared by the method of White *et al.*²⁾ with modifications made mainly to minimize amylolytic activity. The ammonium sulfate precipitate (688 mg protein from 110 g of rabbit muscle) was dissolved in 20 ml of 5 mM Tris-HCl buffer (pH 7.2) containing 0.5 mM EDTA and 10 mM 2-mercaptoethanol (EM-buffer). After dialysis against EM-buffer followed by centrifugation, the solution was applied

Fig. 2. Effect of pH on the amylo-1,6-glucosidase activities.

The activities were assayed on the basis of the hydrolysis of glucosyl β -cyclodextrin (—) and the glucose incorporation into glycogen (---). $\circ$ and $\bullet$, citrate-phosphate buffer; $\triangle$ and $\blacktriangle$, glycine-NaCl-NaOH buffer.

Fig. 3. Effect of the concentration of glucosyl β -cyclodextrin on the amylo-1,6-glucosidase activity.

to a column of ω -aminoethyl Sepharose 4B (1.5 $\times$ 17 cm) preequilibrated with EM-buffer. The column was washed with EM-buffer and then eluted with a linear gradient of 0–0.2 M NaCl in EM-buffer. To the pooled fractions showing more than one-fourth the highest activity was added solid ammonium sulfate (50% satura-

Table 1. The amylo-1,6-glucosidase activities of the purified, partially purified and crude enzyme preparations from rabbit muscle.

Enzyme preparation	Activity, U/mg of protein		Activity ratio P/I
	Glucose production (P) ^{a)}	Glucose incorporation (I) ^{b)}	
Purified (2nd ω -aminoethyl Sepharose)	16.0	0.023	696
Partially purified (Ammonium sulfate, 40% sat.)	0.41	0.00059	695
Crude ^{c)}	2.56 ^{d)}	0.00402 ^{d)}	637

^{a),b)} Assayed on the basis of the hydrolysis of glucosyl β -cyclodextrin and the reverse reaction (glucose incorporation into glycogen), respectively. ^{c)} The crude enzyme was prepared as previously reported.³⁾ ^{d)} U/g of tissue.

tion). The precipitate obtained on centrifugation was dissolved in EM-buffer (4 ml). The solution was applied to a column of Bio-Gel P-150 (3 $\times$ 25 cm) and eluted with EM-buffer. The main fractions around the void volume, which showed no amylolytic activity, were applied to a ω -aminoethyl Sepharose 4B column, and the enzyme was eluted as described above. The elution profiles for both activity and protein showed a single peak. The enzyme (6.7 mg, 26% yield) precipitated with solid ammonium sulfate (50% saturation) showed a specific activity of 22.8 mU per mg of protein, and negligible phosphorylase¹¹⁾ and amylolytic¹²⁾ activities.

Methods. The amylo-1,6-glucosidase assay based on the [¹⁴C] glucose incorporation was carried out according to Nelson and Larner,³⁾ and one unit was defined as 1 μ mol of glucose incorporated per min at 30°C. Protein was determined by the method of Lowry *et al.*¹³⁾ with bovine serum albumin as the standard. The glucose anomer was determined by the method of Okuda *et al.*,¹⁴⁾ except that the reaction pH was 7.0.

Assay for amylo-1,6-glucosidase using glucosyl β -cyclodextrin. The enzyme (5 μ l), which was diluted with 50 mM maleate buffer (pH 6.3) containing 5 mM EDTA, 10 mM 2-mercaptoethanol and 0.5 mg of gelatin per ml, was incubated with 20 μ l of 50 mM glucosyl β -cyclodextrin in 25 mM maleate buffer (pH 6.3) for 15 min at 37°C. After heating in a boiling water bath for 30 sec, the mixture was incubated with N-ethylmaleimide (1.33 mM, 75 μ l)

at room temperature for 30 min. Then to the mixture was added the glucose assay solution (200 μ l),¹⁵⁾ which was prepared by dissolving glucose oxidase (600 U), peroxidase (300 U) and *o*-dianisidine HCl (20 mg) in 100 ml of 0.3 M Tris-HCl buffer (pH 7.0) containing glycerol (40%, v/v). After 1 hr incubation at 37°C, the reaction was terminated by the addition of 18 N H₂SO₄ (200 μ l), and then the resultant color was measured at 540 nm. The amount of glucose formed was calculated using 45 nmol of glucose as a standard. One unit was defined as 1 μ mol of glucose formed per min.

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グルコシル β -サイクロデキストリンを
基質とするアミロ-1,6-グルコンダーゼの
活性測定法

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グルコシル β -サイクロデキストリン (G- β CD) を基質とするアミロ-1,6-グルコンダーゼ活性の分光光度計を用いる測定法を検討した。本方法は、G- β CD から本酵素によって生じるグルコースをグルコースオキシダーゼとペルオキシダーゼを用いて測定するものである。G- β CD に対する本酵素の反応の最適 pH は 6.3 で K_m は 8.3 mM であった。また、生じたグルコースは β -アノマーであった。ウサギ筋肉の酵素標品を本方法で測定した結果は、 ^{14}C -グルコースを用いる逆反応による測定法の結果とよく一致した。