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Note

Enzymic synthesis of *p*-nitrophenyl α-glucobiosides by use of native and immobilized rice α-glucosidase

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INTRODUCTION

α-Glucosidase is known to catalyze not only the hydrolysis of the α-glucosidic linkage in various substrates but also to carry out transglucosylation reactions. In general, plant α-glucosidases have been known to act on malto-oligosaccharides and soluble starch, but little or not at all on synthetic α-glucosides and heterosaccharides such as sucrose and turanose. Plant α-glucosidases act slowly on phenyl α-D-glucoside or *p*-nitrophenyl α-D-glucoside (PNP α-glucoside). However, phenyl α-maltoside is hydrolyzed at a rapid rate[1-6] to give D-glucose and phenyl α-glucoside. It is thus interesting to examine substrate specificity with various synthetic α-glucobiosides with a view of learning whether this is strongly influenced by the position of the intersugar linkage.

The transglycosylation activity of glycosidases has frequently been used for the synthesis of di- or tri-saccharides[7,8]. However, the isolation of the products from the transglycosylation reaction mixture is generally very complicated, because the incubation solution contains starting materials, reaction products, and hydrolyzed materials. When a synthetic glucoside such as PNP α-glucoside is used as an acceptor, it is easy to separate the transglucosylation products from the reaction mixture by the use of an adsorbent such as Amberlite XAD-4 resin, and to determine the individual products quantitatively with high performance liquid chromatography (h.p.l.c.)

In this Note, we describe the enzymic synthesis of PNP α-isomaltoside by the incubation of a reaction system containing a high concentration of maltose as a donor, PNP α-glucoside as an acceptor, and commercially available rice α-glucosidase. We also report the preparation of PNP α-nigeroside and PNP α-maltoside besides PNP α-isomaltoside by the addition of a high concentration of organic solvents to this reaction system, or by the immobilization of the enzyme.

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EXPERIMENTAL

Materials. — Commercial preparations of rice-seed α-glucosidase (EC 3.2.1.20) were purchased from Seikagaku Kogyo Co. (100611, 29 units mg^{-1}) and Sigma Chemical Co. (G-9259, 70 units mg^{-1}). Chitopearl BCW-3507, which is porous chitosan beads produced by Fuji Spinning Co., was used as the carrier for the immobilization of the enzyme. *p*-Nitrophenyl α-D-glucoside (PNP α-glucoside) and PNP α-maltoside were purchased from the Sigma Chemical Co.

Chromatography. — H.p.l.c. was performed under the following conditions: column, Cosmosil $5C_{18}$, 4.6 × 150 mm; solvent system, 20:80 (v/v) acetonitrile–water; flow rate, 0.5 mL min^{-1}; detection, u.v. spectrophotometry at 295 nm.

Assay of α-glucosidase. — A reaction mixture containing 0.05 mL of 0.2M acetate buffer (pH 5.0), 0.10 mL of 0.05M maltose solution, and 0.05 mL of enzyme solution was incubated at 37° for 15 min. The released D-glucose was determined by the D-glucose oxidase–peroxidase method using commercially available Glucose B-test Wako (Wako Pure Chemical Industries). The activity that will hydrolyze 1 μmol of substrate per min under these conditions is defined as 1 unit.

Immobilization of α-glucosidase. — Chitopearl BCW-3507 was washed with deionized water and equilibrated with 2.8M ammonium sulfate solution (pH 5.0). One gram of the Chitopearl was mixed with 290 units of α-glucosidase (Seikagaku Kogyo Co.) suspended in 9 mL of 2.8M ammonium sulfate solution (pH 5.0). The suspension was gently shaken for 2 h at 27°. After centrifugation for 10 min at 2000 r.p.m., the enzyme activity of the supernatant was assayed. The immobilized enzyme was washed with 0.02M and finally 0.05M acetate buffer (pH 5.0).

Assay of immobilized enzyme. — A reaction mixture containing 0.2 mL of 0.03M maltose solution, 0.2 mL of 0.1M acetate buffer (pH 5.0), and 5 mg of immobilized enzyme in a final volume of 0.5 mL was incubated for 10 min at 37° with shaking. The released D-glucose was determined with Glucose B-test Wako as described above. The immobilized enzyme exhibited an activity of 240 units g^{-1}, which corresponded to 83% of the added activity.

Properties of immobilized enzyme. — The optimum pH of the immobilized glucosidase was determined as relative activity, using a series of buffers in the pH range 2.5–9.0. For determining the thermostability of the enzyme, the reaction mixture, complete except for substrate, was treated for 30 min at various temperatures. After cooling, 0.2 mL of substrate solution was added, and the remaining activity was determined by incubation for 10 min at 37°.

Transglucosylation reaction. — *(a) Using the native enzyme.* A reaction mixture containing 0.4 mL of 0.25% PNP α-glucoside (finally 0.1%) dissolved in distilled water or organic solvents, 0.2 mL of 50% maltose solution (finally 10%), 0.2 mL of 0.2M acetate buffer (pH 5.0), and 4 units of enzyme solution in a final volume of 1 mL was incubated at 37°, and intermittently analyzed by h.p.l.c.

(b) Using immobilized enzyme. A reaction mixture containing 0.5 mL of 0.5% PNP α-glucoside (finally 0.1%) dissolved in distilled water or organic solvents, 0.5 mL

of 50% maltose solution (finally 10%), 0.5 mL of 0.2M acetate buffer (pH 5.0), and 100 mg of immobilized enzyme in a final volume of 2.5 mL was incubated at 37° with shaking, and intermittently analyzed by h.p.l.c.

Preparation of transglucosylation products. — *(a) By the batch method.* The reaction was carried out in 0.05M acetate buffer (100 mL, pH 5.0) containing PNP α-glucoside (100 mg) and maltose (10 g), by incubation with 290 units of rice α-glucosidase for 12 h at 37°. The reaction mixture was applied to a column of Amberlite XAD-4 (1.5 × 15 cm) and eluted with methanol. The eluate was concentrated and chromatographed on a Toyopearl HW-40S column (2 × 110 cm) with 1:4 methanol–water as eluant to give PNP α-isomaltoside in a yield of 24% (based on the amount of PNP α-glucoside added as an acceptor). The addition of 40% (v/v) methanol to the reaction mixture just described, followed by the chromatographic separation of the products in a similar manner, gave PNP α-isomaltoside, PNP α-nigeroside, and PNP α-maltoside in yields of 13, 8, and 4%, respectively. A mixture of the separated PNP α-glucobiosides was analyzed by h.p.l.c. (Fig. 2), the retention time of peak 2 being in good agreement with that of commercial PNP α-maltoside.

(b) By the continuous method. Immobilized enzyme (290 units, 1.2 g) was packed into a column (10 × 30 mm). A 0.05M acetate buffer solution (100 mL, pH 5.0) containing PNP α-glucoside (100 mg) and maltose (10 g) was circulated through the column by a peristaltic pump at room temperature. After 48 h, the solution was treated with Amberlite XAD-4 resin and chromatographed on a Toyopearl HW-40S column (2 × 110 cm) to give PNP α-isomaltoside, PNP α-nigeroside, and PNP α-maltoside in yields of 15, 8, and 4%, respectively. After reaction, the column was washed with 0.05M acetate buffer (pH 5.0) and reused.

N.m.r. analysis. — ^{13}C-N.m.r. spectra (25.0 MHz) of samples in deuterated dimethyl sulfoxide were recorded with a JEOL FX-100 spectrometer.

RESULTS

The time course of the transglucosylation reaction of rice α-glucosidase acting on maltose and PNP α-glucoside is shown in Fig. 1. The formation of PNP α-isomaltoside as the main transglucosylation product in aqueous medium, and the decay of this product on further incubation, is traced in panel *a*. The addition of methanol to the reaction mixture decreased the formation of PNP α-isomaltoside, but PNP α-nigeroside and PNP α-maltoside appeared as products. The proportions of PNP α-nigeroside and PNP α-maltoside increased with the addition of up to 40% (v/v) methanol (panels *b* and *c*). However, the addition of 60% methanol to the reaction mixture completely inhibited the transglucosylation reaction (data not shown). When acetonitrile was added in place of methanol a more abundant accumulation of PNP α-nigeroside and PNP α-maltoside was observed (panel *d*). No difference was seen in the course of the transglucosylation reaction catalyzed by the two rice α-glucosidase preparations, obtained from two different commercial sources.

PNP α-glucobiosides isolated as described in the Experimental section were

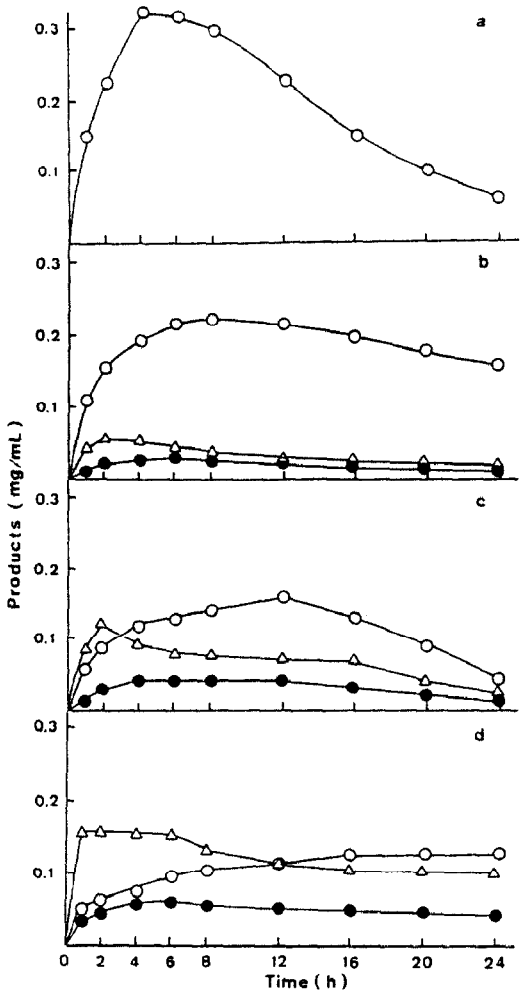

Fig. 1. Time course of the transglucosylation reaction of rice α-glucosidase with maltose and PNP α-glucoside as substrates. Reaction mixtures were incubated at 37° without organic solvent (a), or in the presence of 20% methanol (b), 40% methanol (c), or 40% acetonitrile (d). Symbols: ○, PNP α-isomaltoside; △, PNP α-nigeroside; ●, PNP α-maltoside.

analyzed by h.p.l.c. (Fig. 2) and ^{13}C-n.m.r. spectroscopy. The ^{13}C-n.m.r. spectrum of each product corresponded to that given in the literature[9].

Recently, commercially available porous chitosan beads have been used as support matrices for the immobilization of glycohydrolases[10–12]. We could efficiently immobilize rice α-glucosidase on Chitopearl BCW-3507, which operates in the hydrophobic binding mode, and obtain high retention of activity (83%). The optimum pH's of the native and immobilized enzymes were 5.0 (ref. 13) and 4.0, respectively. Immobilized enzyme retained higher activity at acidic pH (below 4.0). The thermostability of the enzyme was also improved by immobilization, as shown in Fig. 3.

It is very interesting that the transglucosylation products formed by the native

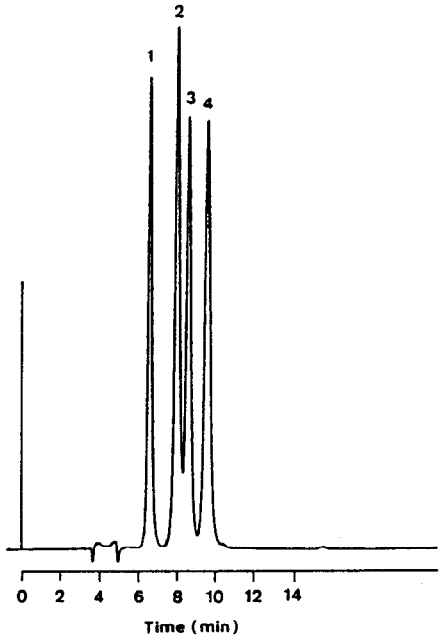

Fig. 2. H.p.l.c. of a reconstituted mixture of PNP α-glycosides: 1, PNP α-isomaltoside; 2, PNP α-maltoside; 3, PNP α-nigeroside; 4, PNP α-glucoside.

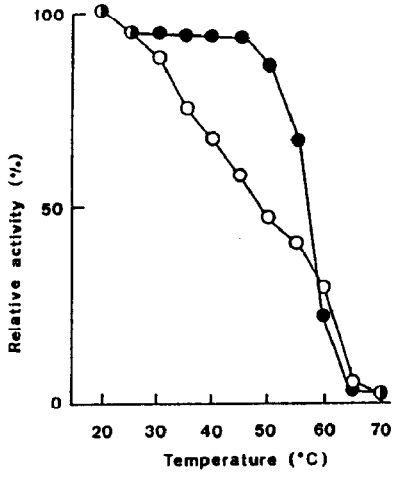

Fig. 3. Thermostability of native and immobilized enzymes. Symbols: ○, native enzyme; ●, immobilized enzyme.

("free") enzyme only in the presence of organic solvents were formed by the immobilized enzyme in a purely aqueous medium (Fig. 4). The addition of 40% methanol to the reaction mixture using the immobilized enzyme decreased the formation of PNP α-isomaltoside, but had little effect on the formation of PNP α-nigeroside and PNP α-maltoside. When 40% acetonitrile instead of methanol was added to the reaction

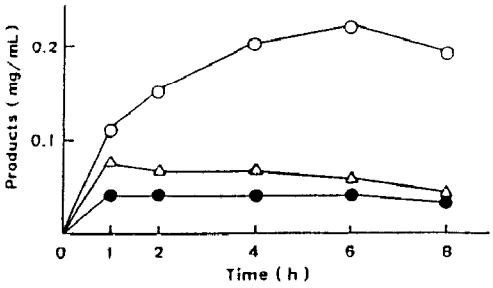

Fig. 4. Time course of the transglucosylation reaction of immobilized rice α-glucosidase. The reaction mixture was incubated at 37° with shaking. Symbols: ○, PNP α-isomaltoside; △, PNP α-nigeroside; ●, PNP α-maltoside.

mixture, the formation of PNP α-isomaltoside was markedly depressed, and the formation of PNP α-nigeroside selectively increased, as seen with the native enzyme.

DISCUSSION

α-Glucosidases from fungi have been classified into four groups according to their modes of transglucosylation and their starch-hydrolyzing activity[5]. This classification of α-glucosidases may also be applicable to plant α-glucosidases. Recently, Muto *et al.*[13] reported that commercially available rice α-glucosidase produced maltotriose as the main product of transglucosylation from maltose, and that isomaltose and panose, bearing α-(1→6)-glucosidic linkages, were not detected in the reaction mixture. Consequently, this enzyme can be classified in Group I, enzymes that readily hydrolyze soluble starch and produce maltotriose as the main transglucosylation product from maltose. We found that rice α-glucosidase produces PNP α-isomaltoside as the only product of transglucosylation from maltose as a donor to PNP α-glucoside, although we did not investigate glucosyl transfer to maltose or methanol. This shows that the position-specificity of the enzyme varies with the acceptor provided. The addition of methanol to the reaction mixture improves the solubility of PNP α-glucoside and in some cases results in a great increase in the formation of transglucosylation product[14,15]. As reported by Usui and Murata[14], the addition of methanol in the reaction system may be important in strengthening enzyme–enzyme interaction by changing the enzyme conformation, or in altering the solvation environment so as to increase the hydrophobicity and decrease the dielectric constant on the surface of the enzyme. In this work, the addition of methanol to the reaction mixture did not result in a higher yield of transglucosylation product, but promoted the formation of the new transglucosylation products PNP α-nigeroside and PNP α-maltoside. It should be noted that the total yield of transglucosylation products in the presence of methanol was the same as that of PNP α-isomaltoside in the reaction mixture without organic solvents.

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