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ENZYMIC PREPARATION OF PANOSE AND ISOPANOSE FROM PULLULAN*

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ABSTRACT

The trisaccharides panose and isopanose were prepared in good yield from enzymic hydrolyzates of pullulan. Pullulan was hydrolyzed by the purified alpha amylase preparation of *Thermoactinomyces vulgaris* R-47. The digest was applied to a carbon–Celite column and eluted with a linear gradient of 1-propanol from 0 to 5%. From the trisaccharide fractions eluted, panose was prepared in about 70% yield. Pullulan was also hydrolyzed by purified isopullulanase (EC 3.2.1.57 pullulan 4-glucanohydrolase) of *Aspergillus niger* ATCC-9642, and isopanose was prepared in about 90% yield by using the same technique as that for the preparation of panose.

INTRODUCTION

Panose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranose] and isopanose [*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranose], the D-glucose-containing trisaccharides having (1 → 4)- and (1 → 6)-α-D-glucosidic linkages, have been isolated by many researchers[1–5] from acid hydrolyzates of amylopectin, glycogen, and pullulan. Panose has also been prepared by the action of a transglycosylase from *Aspergillus niger* NRRL-337 on maltose[6], and isopanose has been synthesized by a coupling reaction between isomaltose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranose] and cyclohexaamylose catalyzed by *Bacillus macerans* transglycosylase[7].

Some of the present authors[8–10] have reported on the production and properties of an isopullulanase (EC 3.2.1.57 pullulan 4-glucanohydrolase) that cleaves (1 → 4)-α-D-glucosidic linkages adjacent to the (1 → 6)-α-D-glucosidic linkages of pullulan[11] and panose, to produce isopanose, and isomaltose and D-glucose, respectively. In the course of studies on hydrolysis of pullulan by microbial enzymes, we have found a new type of pullulan-hydrolyzing activity in the purified alpha amylase preparation of an Actinomycetales, *Thermoactinomyces vulgaris* R-47. The action of the enzyme

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

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on pullulan is quite different from that of pullulanase (EC 3.2.1.41 pullulan 6-glucanohydrolase[12–16] as well as isopullulanase; this novel enzyme splits (1 → 4)-α-D-glucosidic linkages of pullulan to produce panose, and not isopanose.

The present paper describes the large-scale preparation of panose and isopanose from hydrolyzates of pullulan by the new enzyme and by isopullulanase, respectively.

EXPERIMENTAL

Enzymes. — Alpha amylase of *Thermoactinomyces vulgaris* R-47 was highly purified by Tamura *et al.*; the method of purification and properties of the enzyme will be described elsewhere. The purified enzyme showed a single band on disc poly(acrylamide)-gel electrophoresis as well as on sodium dodecyl sulfate–poly-(acrylamide)-gel electrophoresis.

Isopullulanase was prepared as described by Sakano *et al.*[9].

Hydrolysis of pullulan. — A. Hydrolysis by T. vulgaris *R-47 alpha amylase.* Pullulan (20 g), prepared from the culture broth of *Aureobasidium pullulans*‡ (syn. *Pullularia pullulans*) S-1 and purified as described by Taguchi *et al.*[17], was dissolved in 2 liters of 60mM acetate buffer (pH 5.0) containing 5mM calcium chloride, and the pullulan solution was incubated with 500 μl of alpha amylase solution (60 units/ml) at 60° under toluene. After incubation for 63 h, 500 μl of the enzyme solution was added to the digest, which was then incubated for another 67 h at 60°. The digest was autoclaved for 15 min at 110° to stop the enzyme reaction, filtered, and concentrated *in vacuo* in a rotary evaporator to a syrup.

B. Hydrolysis by isopullulanase. Pullulan (20 g) was dissolved in 2 liters of 60mM acetate buffer (pH 3.5), and the solution was incubated with 1 ml of isopullulanase (8 units) at 40° under toluene. After incubation for 24 h, 1 ml of the enzyme solution was added to the digest, and incubation was continued for another 24 h at 40°. The digest was autoclaved for 15 min at 110° to stop the enzyme reaction, filtered, and concentrated *in vacuo* in a rotary evaporator to a syrup.

Carbon–Celite column chromatography. — Carbon–Celite column chromatography was performed as described by S. Kobayashi *et al.*[18]. Pullulan hydrolyzates (2 g) were applied to a carbon–Celite column (6.5 × 35 cm) and eluted with a linear gradient (0–5%) of 1-propanol. The flow rate was 200 ml/h and 10-ml fractions were collected automatically.

Analysis of carbohydrate. — Total carbohydrate was determined by the phenol–sulfuric acid method[19]. Reducing sugar was determined by the Nelson–Somogyi method[20]. Paper chromatography was carried out by the method of D. French *et al.*[21]. Paper electrophoresis was performed as described elsewhere[8].

Assay of enzyme activity. — The pullulan-hydrolyzing activity of the purified alpha amylase preparation from *T. vulgaris* R-47 was estimated by determining the

‡*Pullularia pullulans* S-1 was isolated and kindly supplied by Dr. S. Ueda, Kyushu University (see ref 16a.).

reducing sugar produced after incubating the enzyme with 1% aqueous pullulan at pH 5.0 for 30 min at 60°. One unit of the activity is defined as the amount of enzyme that produces reducing sugar equivalent to one μmol of maltotriose per min. Isopullulanase activity was estimated by determining reducing sugar after incubation of the enzyme with 1% aqueous pullulan at pH 3.5 for 30 min at 40°. One unit of isopullulanase activity is defined as described previously[9].

RESULTS AND DISCUSSION

The enzymic digests of pullulan by the alpha amylase of *T. vulgaris* and isopullulanase were separately dissolved in small amounts of water, applied to a carbon–Celite column (6.5 × 35 cm) and eluted with a linear, 0–5% gradient of 1-propanol in water. Eluates were collected in 10-ml fractions and analyzed for carbohydrate content. Typical elution-patterns of products from pullulan are shown in Figs. 1a and 1b. Products 1, 2, 3, 4, and 5 (P1, P2, P3, P4, and P5) were separately pooled, concentrated to syrups, which were dehydrated by repeated addition of methanol, followed, in each case, by evaporation *in vacuo*. The yields of P1, P2, P3, P4, and P5 were 1.43 g, 28 mg, 95 mg, 1.78 g, and 120 mg, respectively.

One mg of P1 and isopullulanase (0.02 unit) were incubated for 3 h at 40° in a final volume of 200 μl (50mM acetate buffer, pH 3.5), and the products of hydrolysis of P1 were identified by paper chromatography as glucose and isomaltose (Fig. 2).

Product P1 (20 mg) was reduced by the method of Wolfrom and Thompson[22] to the corresponding alditol, and acetylated as described previously[1,2]. The acetate

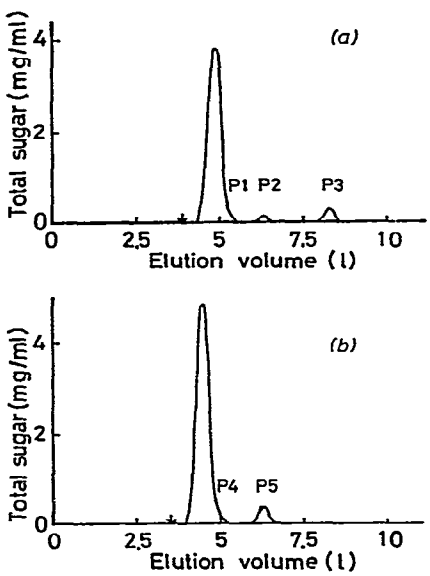

Fig. 1. Carbon–Celite column chromatography of pullulan hydrolyzates (see Experimental section for details). (*a*) Chromatography of products of pullulan hydrolyzed by the alpha amylase preparation of *T. vulgaris* R-47. (*b*) Chromatography of products of pullulan by isopullulanase.

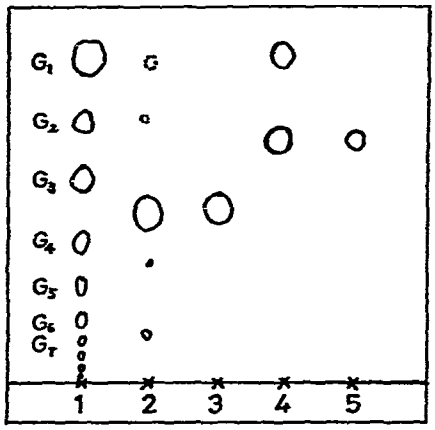

Fig. 2. Paper chromatography of pullulan hydrolyzates by *T. vulgaris* alpha amylase and Pl-hydrolyzates by isopullulanase. Track 1: malto-oligosaccharide series of glucose (G_1), maltose (G_2), and so on, 2: hydrolyzates of pullulan with *T. vulgaris* alpha amylase, after incubation for 140 h, 3: Pl, 4: hydrolyzates of Pl with isopullulanase, and 5: isomaltose.

of the alditol was crystallized from ethanol: yield ~24 mg, m.p. 150–151° (m.p. 148.5–150° of authentic dodecaacetyl-panitol[6]). Paper electrophoresis in 0.01M borate buffer (pH 10.0) showed Pl to be indistinguishable from panose. Thus Pl, the trisaccharide produced from pullulan by the new enzyme, was identified as panose.

From the results described here, it is evident that a new type of pullulan-hydrolyzing activity is present in the preparation of alpha amylase from an Actinomycetales strain; it acts quite differently from pullulanase, isopullulanase, or glucoamylase. The enzyme cleaves (1 → 4)-α-D-glucosidic linkages in pullulan and liberates panose as the main product. The proposed action-pattern of the enzyme in hydrolysis of pullulan is shown in Fig. 3. Use of the enzyme facilitates the larger-scale preparation of panose from pullulan, in comparison with the tedious methods described previously[1-6]. By using the new enzyme, panose was prepared from pullulan in about 70% yield.

Fraction P4 (20 mg) was reduced and acetylated by the same method described earlier. The acylated alditol of P4 crystallized from ethanol; yield ~25 mg; m.p. 70–71° (m.p. 69–72° of authentic dodecaacetyl-isopanitol[4]). Paper electrophoresis (0.01M borate buffer, pH 10.0) of P4 has been described previously[8], and it indicated that P4 was isopanose. Therefore, it was confirmed that P4, the trisaccharide produced from pullulan by isopullulanase, is isopanose.

Isopullulanase may be used to facilitate a larger-scale preparation of isopanose

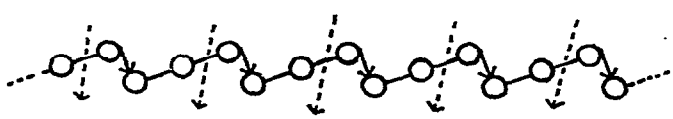

Fig. 3. Cleavage of pullulan by the new pullulan-hydrolyzing enzyme. ○: glucose, —: (1→4)-α-D-glucosidic linked, ↓: (1→6)-α-D-glucosidic linked, ↙: attack points.

from pullulan, as compared with acid hydrolyses of amylopectin and pullulan[4,5], and the coupling reaction of isomaltose and cyclohexaamylose[7]. By using isopullulanase, isopanose was prepared from pullulan in about 90% yield.

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