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HYDROLYSIS OF LOW MOLECULAR WEIGHT ISOMALTOSACCHARIDES BY A *p*-NITROPHENYL-α-D-GLUCOPYRANOSIDE-HYDROLYZING α-GLUCOSIDASE FROM A THERMOPHILE, *BACILLUS THERMOGLUCOSIDIUS* KP 1006

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Summary

A *p*-nitrophenyl-α-D-glucopyranoside-hydrolyzing α-glucosidase of a thermophile, *Bacillus thermoglucosidius* KP 1006, was purified to an electrophoretically-homogeneous state. Its molecular weight was estimated as 60 000 by gel electrophoresis. The molecular activity (k_0) and the K_m value at 60°C and pH 6.8 for *p*-nitrophenyl-α-D-glucopyranoside were 233 s^{-1} and 0.24 mM, respectively. The enzyme cleft the non-reducing terminal α-1,6-glucosidic bonds of isomaltose, panose, isomaltotriose, isomaltotetraose, and isomaltopentaose. The k_0 values were 72.4, 194, 208, 233 and 167 s^{-1}, and the K_m values were 3.3, 9.5, 11, 13 and 21 mM, respectively. Each isomaltosaccharide was hydrolyzed to glucose by the cleavage of single glucose units from its non-reducing end. The present study suggests that the enzyme is an oligo-1,6-glucosidase (dextrin 6-α-glucanohydrolase, EC 3.2.1.10) and an exo-glucosidase.

Introduction

A thermophilic organism *Bacillus thermoglucosidius* KP 1006 can synthesize an extracellular, thermostable α-glucosidase responsible for rapid hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside [1]. The purified enzyme is unique in its narrow substrate specificity, since the enzyme acts effectively only on isomaltose among the naturally occurring sugars tested [2]. The present study demonstrates that the homogeneously purified α-glucosidase can split the non-reducing terminal α-1,6-glucosidic linkages involved in panose and isomaltosaccharides with chain length $n = 2$—5 glucose units. Also, the K_m and the molecular activity (k_0, maximum velocity divided by molar concentration of enzyme) for each substrate are presented. On the basis of its substrate specific-

ity and action pattern, *B. thermoglucosidius* α-glucosidase has been assigned to oligo-1,6-glucosiase (dextrin 6-α-glucanohydrolase, EC 3.2.1.10) [1—5].

Materials and Methods

Materials. Isomaltosaccharides (n = 3—5) were prepared by acid hydrolysis of dextran according to the method of Bailey and Clarke [6]. Gel filtration of each sugar on a Sephadex G-25 column (4.4 × 98 cm) eluted with water (flow rate 0.21 ml/min) was repeated until the sample was purified to paper chromatographical homogeneity. The amounts of isomaltosaccharides were determined by the reductometric method with 3,5-dinitrosalicyclic acid [7]. Panose, nigerose and kojibiose were kindly supplied by Dr. K. Matsuda, Department of Agricultural Chemistry, Tohoku University, Sendai. Hog stomach mucosa pepsin and horse heart cytochrome *c* were purchased from Sigma Chemical Co., and beef pancreatic α-chymotrypsinogen from Miles Laboratories. Other chemicals and reagents used were the same as described previously [1,2].

Enzyme. B. thermoglucosidius α-glucosidase prepared by the method of Suzuki et al. [2] was further purified to obtain an electrophoretically homogeneous sample as follows. The enzyme preparation [15 ml, 18 mg protein; spec. act. 173 units/mg protein (1 unit, defined as the amount of enzyme needed for hydrolysis of 1 μmol *p*-nitrophenyl-α-D-glucopyranoside per min at 60°C and pH 6.8 [2])] was dialyzed against 50 mM potassium phosphate (pH 7.5), 1 mM EDTA (Buffer A) and then chromatographed on a DEAE-Sephadex A-50 column (1.8 × 23.4 cm, previously equilibrated with Buffer A) with 2 l linear gradient 0.1—0.4 M NaCl in Buffer A (flow rate 0.33 ml/min; 10-ml fractions). Active fractions at 0.15—0.18 M NaCl were pooled. This chromatography was repeated once more using the DEAE-Sephadex eluate. The enzyme solution from the previous step was concentrated by ultrafiltration in a collodion bag, and filtered through a Sephadex G-100 column (1.6 × 98.5 cm) with 0.15 M potassium phosphate (pH 6.8), 1 mM EDTA (flow rate 0.32 ml/5 min; 1.6-ml fractions). Elution profile of the enzyme activity was coincident with that of protein; active fractions were combined (1.36 mg protein. specific activity 245 units/mg protein). This preparation gave a single protein band in disk electrophoresis on polyacrylamide gel [2,8]. The molecular weight (M_r) of the enzyme was estimated as 60 000 * by SDS-gel electrophoresis by the method of Weber and Osborn [9], using the following standards: bovine serum albumin (M_r = 68 000), egg albumin (45 000), hog stomach mucose pepsin (35 000), beef pancreatic α-chymotrypsinogen (23 000), equine skeletal muscle myoglobin (17 800) and horse heart cytochrome *c* (13 400).

Assay. p-Nitrophenol release from *p*-nitrophenyl-α-D-glucopyranoside by the action of α-glucosidase was followed photometrically [2]. Glucsoe formed from isomaltosaccharides, panose, nigerose and kojibiose was measured with a

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* This agreed with the value achieved by the gel filtration on Bio-gel P-150 (Suzuki, Y. and Nakamura, N., unpublished data), but not with that obtained on Sephadex G-200 (M_r = 55 000) [2]. The higher value was used for determination of k_0.

Beckman oxygen electrode and glucose oxidase [2]. The standard reaction system contained in 0.5 or 1 ml total volume: 33.3 mM potassium phosphate (pH 6.8), 23.6 nM enzyme, and 10 mM substrate (when *p*-nitrophenyl-α-D-glucopyranoside was used as substrate, the enzyme level was fixed at 2.21 nM). Incubation was at 60°C for 1—10 min. The enzyme concentration was assessed by the turbidimetric method of Robenbloom et al. [10].

Results

Liberation of non-reducing terminal α-1,6-linked glucose residues of panose and isomaltosaccharides

α-Glucosidase released glucose from panose and isomaltosaccharides with chain length n = 2—5. The reaction proceeded under a linearity during incubation for 10 min. At the end of the reaction, the paper chromatography showed only two products in the digest of each sugar except isomaltose (Fig. 1). One corresponded to glucose, which was the sole product from isomaltose. The other agreed with maltose from panose, or with the isomaltosaccharide one glucose unit smaller than added substrate. The hydrolysis of panose indicates that the linkage to be split in each isomaltosaccharide is the non-reducing terminal one.

K_m and k_0

Table I lists the K_m values for panose and isomaltosaccharides (n = 2—5), and the k_0 values for the non-reducing terminal α-1,6-linkage hydrolysis of respective sugars, together with those of *p*-nitrophenyl-α-D-glucopyranoside. This

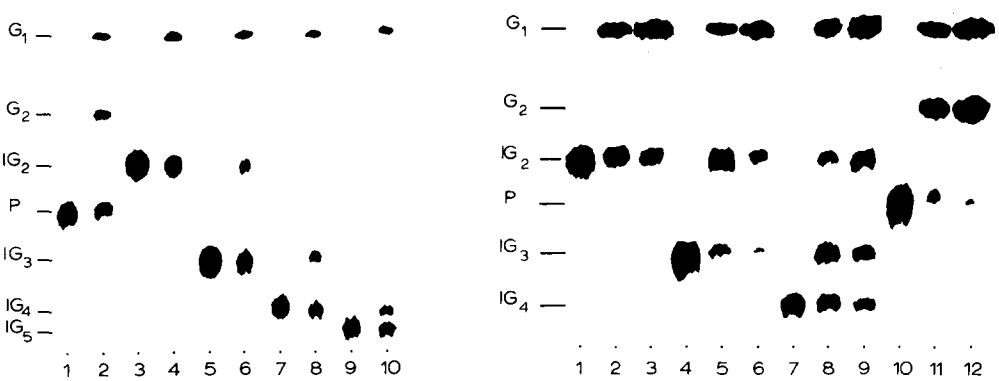

Fig. 1. A paper chromatogram of the product from panose and isomaltosaccharides at 0 min (1, 3, 5, 7 and 9) or 10 min (2, 4, 6, 8 and 10) of α-glucosidase reaction. 10-μl aliquots of the reaction mixtures (1—10) were applied on a Tôyô No. 51 filter paper. Five ascents of 23.5 cm each were made with *n*-butanol/pyridine/water (6 : 4 : 3, v/v) as solvent, and the spots detected using silver nitrate as described by Welker and Campbell [11]. Substrates used: 1 and 2, panose; 3 and 4, isomaltose; 5 and 6, isomaltotriose; 7 and 8, isomaltotetraose; 9 and 10, isomaltopentaose. P, panose; G_1, glucose; G_2, maltose; IG_2, isomaltose; IG_3, isomaltotriose; IG_4, isomaltotetraose; IG_5, isomaltopentaose.

Fig. 2. Hydrolysis of isomaltose (1—3), isomaltotriose (4—6), isomaltotetraose (7—9) and panose (10—12) during a 1 or 2 h incubation with α-glucosidase. 20-μl portions of the reaction mixture were taken at 0 h (1, 4, 7 and 10), 0.5 h (8), 1.0 h (2, 5, 9 and 11), and 2.0 h (3, 6 and 12) and subjected to paper chromatography, as described in Fig. 1. The abbreviations are the same as in Fig. 1.

TABLE I

THE K_m VALUES AND THE MOLECULAR ACTIVITIES (k_0) OF α-GLUCOSIDASE FOR ISOMALTOSACCHRIDES, PANOSE, AND *p*-NITROPHENYL-α-D-GLUCOPYRANOSIDE

The initial rates (v) of glucose liberation were determined over 1—10 mM panose, 4—22 mM isomaltosaccharide, or 0.1—2.0 mM *p*-nitrophenyl-α-D-glucopyranoside levels. The K_m and V values were obtained by plotting [S]/v vs. [S] [12], or 1/v vs. 1/[S] [13]. The k_0 was calculated from V.

Substrate	K_m (mM)	k_0 * (s^{-1})	k_0/K_m ($s^{-1} \cdot mM^{-1}$)
p-Nitrophenyl-α-D-glucopyranoside	0.24	233 **	971
Isomaltose	3.3	74.2	22.5
Panose	9.5	194	20.4
Isomaltotriose	11	208	18.9
Isomaltotetraose	13	233	17.9
Isomaltopentaose	21	167	8.0

* Expressed as non-reducing terminal α-1,6-bond hydrolysis.

** As glucosidic linkage hydrolysis.

synthetic substrate gave the lowest K_m and the largest k_0 (equal to k_0 of isomaltotetraose). Of isomaltosaccharides, both rate parameters were minimum for isomaltose, Each parameter increased about 3 times between isomaltose and isomaltotriose. Panose, isomaltotriose and isomaltotetraose had almost similar K_m and k_0 values. The elongation of chain length up to $n = 5$ caused a rise in K_m and fall in k_0.

Exo-type action

Isomaltosaccharides ($n = 2$—4) and panose were allowed to react with α-glucosidase for 1 or 2 h, respectively. The products were analyzed by paper chromatography at defined intervals. Glucose from these sugars accumulated continuously as the reaction proceeded. Maltose production from panose was parallel with glucose release, and isomaltose formation was not detectable. For the first hour of isomaltotriose hydrolysis, isomaltose accumulated, but its content fell at 2 h incubation. A significant amount of isomaltotriose, and a trace of isomaltose were detected in the digest of isomaltotetraose at 30 min reaction. However, isomaltotriose decreased within 30 min after this time, while isomaltose increased.

Weak activity for kojibiose and nigerose

Kojibiose and nigerose underwent 0.37 and 0.06% hydrolysis, respectively, by α-glucosidase after a 10 min incubation (cf. isomaltose 3.8% hydrolysis). Glucose was identified as the only product from each sugar by paper chromatography.

Discussion

Among the substrates which have examined, *p*-nitrophenyl-α-D-glucopyranoside is the most effective for *B. thermoglucosidius* α-glucosidase [2] (Table I). Although maltose can be hydrolyzed as well as the aryl glucoside by a number

of bacterial and yeast α-glucosidases [14—18], the thermophile enzyme fails to attack this disaccharide at all [2].

The activity of purified *B. thermoglucosidius* α-glucosidase is restricted only towards the non-reducing terminal α-1,6-linkages involved in panose and short-chain isomaltosaccharides among the naturally occurring sugars tested, although α-1,3-bond of nigerose and α-1,2-bond of kojibiose are slightly split, as described above [2]. The enzyme is an exo-glucosidase, catalyzing the complete hydrolysis of isomaltosaccharide into glucose by the cleavage of single glucose units from its non-reducing end. This seems justified by the following observations: (i) the α-1,6-linkage of panose can be hydrolyzed, which locates at the non-reducing end of the molecule (Fig. 1 and 2); (ii) glucose plus isomaltotriose, and glucose plus isomaltotetraose are rapidly released from isomaltotetraose and isomaltopentaose, respectively (Fig. 1); and (iii) during a complete hydrolysis of isomaltotetraose, isomaltose accumulation is preceded by isomaltotriose accumulation, and isomaltose increases in its content concomitantly with isomaltotriose disappearance (Fig. 2).

B. thermoglucosidius α-glucosidase strongly resembles oligo-1,6-glucosidases extracted from *Streptococcus mitis* [3], *Lactobacillus bifidus* [4], and hog intestinal mucosa [5,19,20] regarding its substrate specificity, although all of these mesophile enzymes have never been purified homogeneously. Also, it is not clear whether the mesophile oligo-1,6-glucosidases are associated with the *p*-nitrophenyl-α-D-glucopyranoside-hydrolyzing activity.

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