Journal of Industrial Microbiology, 7 (1991) 251–256
Elsevier

SIM 00341

Purification and properties of β-fructofuranosidase from *Aureobasidium* sp. ATCC 20524

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(Received 1 December 1990; revised 28 January 1991; accepted 4 February 1991)

Key words: Fructooligosaccharide; 1-Kestose; Glycoprotein; Fructosyl-transferring activity

SUMMARY

Purification and properties of two β-fructofuranosidases, which produce 1-kestose (1^F-β-fructofuranosyl-sucrose) from sucrose, from *Aureobasidium* sp. ATCC 20524 are reported. The enzymes were purified to homogeneity by fractionations involving ethanol, calcium acetate and ammonium sulfate and DEAE-Cellulofine and Sephadex G-200 chromatography. Molecular weights of the enzymes were estimated to be about 318000 (*P*-1) and 346000 (*P*-2) daltons by gel filtration. The enzymes were glycoproteins that contained about 30% (w/v) (*P*-1) and 53% (w/v) (*P*-2) carbohydrate. The optimum pH for the enzymatic reactions were 4.5–5.5 (*P*-1) and 4.5–6 (*P*-2). The enzymes were stable over a wide pH range (4–9). The optimum reaction temperatures for both enzymes were 50–55 °C and they retained more than 94% (*P*-1) and 98% (*P*-2) activities at 50 °C after 15 min. The K_m values for sucrose were 0.47 M (*P*-1) and 0.65 M (*P*-2). The enzymes were inhibited by mercury, copper and lead ions as well as *p*-chloromercuribenzoate.

INTRODUCTION

Investigations about fructosyl-transferring enzymes that produce fructooligosaccharides have become very important because their products have favorable functional properties (increase of *Lactobacillus bifidus*, indigestibility, diminution of dental caries) for foods [7,12]. Two microbial β-fructofuranosidases, from *Penicillium* sp. [9] and from *Aspergillus niger* [8], that have high fructosyl-transferring activities have been purified and their enzymatic characteristics have been reported.

In the present report, we describe the purification and properties of two β-fructofuranosidases from *Aureobasidium* sp. ATCC 20524 which produce 1-kestose from sucrose [5,6]. The enzymatic reactions are described in Fig. 1.

MATERIALS AND METHODS

Microorganism

Aureobasidium sp. ATCC 20524 was maintained on agar slants (sucrose 1%, yeast extract 0.2%, agar 1.8%, pH 5–5.5) for this investigation.

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sucrose ⟶ 1-kestose + glucose (1)

sucrose ⟶ fructose + glucose (2)

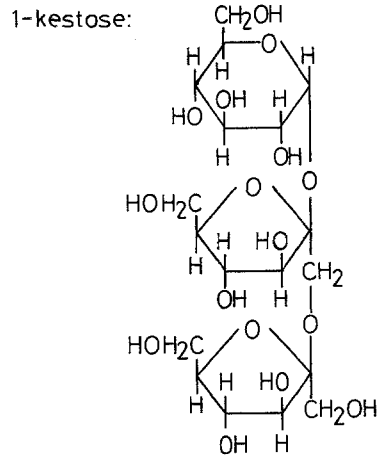

Fig. 1. The reactions of enzymes from *Aureobasidium* sp. ATCC 20524. Both enzymes have strong fructosyl-transferring activity (1) and very weak sucrose-hydrolysing activity (2). The reaction (1) is observed at the reaction condition described in Materials and Methods. Addition of hydrolysing reaction to transferring reaction is observed at 40 °C after 48 h.

Cultivation and preparation of crude enzyme

Cultivation of the strain for enzyme production was carried out in liquid culture (sucrose 20%, yeast extract 2%, $NaNO_3$ 1%, K_2HPO_4 0.75%, $MgSO_4 \cdot 7\,H_2O$ 0.1%, pH 6.5–7) at 30 °C for 2 days with the same conditions described in a previous report [6]. Cells were harvested by centrifugation and lyophilised. The enzymes were solubilised from 5 g of dried cells by 20 mg of Kitalase (2000 U endo-β-1,3-glucanase/g, Wako), which has no β-fructofuranosidase activity, in 75 mM McIlvain buffer (pH 5) at 40 °C for 2 h and then centrifuged. The supernatant was used for enzyme purification.

Purification of the enzyme

Two volumes of ethanol were added to the crude enzyme solution. The resultant precipitate was collected and dissolved in distilled water. To the solution, $(CH_3COO)_2Ca$ was added to 6% (w/v) and then centrifuged. The supernatant was dialysed against 0.02 M Tris-HCl buffer (pH 8). To the dialysate, $(NH_4)_2SO_4$ was added to 100% saturation and then centrifuged. The supernatant was collected by centrifugation and then dialysed against 20 mM Tris-HCl buffer (pH 8). The above procedures were carried out on ice.

The dialysate was applied on a DEAE-Cellulofine A-800 column (0.9 × 30 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8). The column was washed with the same buffer and then eluted stepwise by the same buffer which contained 0.025, 0.05, 0.07 and 0.09 M NaCl. The fractions were collected and concentrated with a membrane filter (Amicon UM 10).

The enzyme solution from the previous stage was applied on a Sephadex G-200 column (1.5 × 87 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8) containing 0.1 M NaCl. After application of the enzyme to the column, it was eluted with the same buffer at a flow rate of 10 ml/h. The above chromatographies were carried out at 20 °C.

Molecular weight estimation

The molecular weight of the enzymes was estimated by Sephadex G-200 (1.5 × 87 cm) gel filtration [1]. The column was equilibrated with 20 mM Tris-HCl buffer (pH 8) containing 0.1 M NaCl. After application of the purified enzyme on the column, it was eluted with the same buffer at a flow rate of 10 ml/h. The elution of standard protein (ferritin, catalase, aldolase and bovin serum albumin, Boehringer-Mannheim) was carried out in the same manner.

Electrophoresis

Disc-electrophoresis of the purified enzymes was performed at a constant current of 3 mA per gel on a 3.75% (w/v) polyacrylamide gel at pH 8.3 [2]. After electrophoresis, the gel was stained with Coomassie blue.

Assays

Enzyme activity. The reaction mixture consisted of 0.1 ml of enzyme solution, 0.4 ml of 75% (w/v) sucrose, 0.5 ml of 0.15 M McIlvain buffer (pH 5.0), at 50 °C for 20 min [6].

Products in the reaction mixture were measured by HPLC (Shimadzu LC-4A) fitted with a μ-Bondapack CH (3.9 × 30 mm, Waters) column under the following conditions: temperature, 27 °C; mobile phase, acetonitrile/water (77 : 23, v/v); flow rate, 1 ml/min; and RI detector. Released amount of glucose in the reaction mixture was assayed by the glucose oxidase method (Wako glucose test B). One unit of enzyme activity was defined as the quantity of enzyme responsible for the transfer of 1 μmol of fructose in 1 min.

Protein. Protein was assayed with bovine serum albumin (Nakarai) as standard [10].

Carbohydrates. Carbohydrates were assayed with glucose (Wako) as standard [3].

RESULTS AND DISCUSSION

Purification of the enzyme

The results of purification of β-fructofuranosidase from *Aureobasidium* sp. ATCC 20524 are shown in Table 1. The two β-fructofuranosidase enzymes (*P*-1 and *P*-2) were separated from each other by DEAE-Cellulofine A-800 ion exchange column chromatography (Fig. 2). The

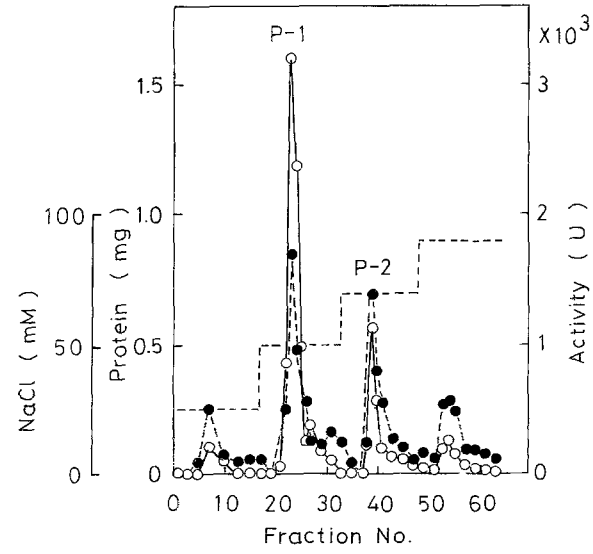

Fig. 2. DEAE-Cellulofine A-800 column chromatograph of the enzymes from *Aureobasidium* sp. ATCC 20524. Symbols: enzymatic activity, ○; protein, ●.

TABLE 1

Purification of β-fructofuranosidase from *Aureobasidium* sp. ATCC 20524

Step	Activity (U)	Protein (mg)	Specific activity (U/mg protein)	Yield (%)
Crude extract	127736	3992	32.0	100
Ethanol	102188	1504	67.9	80
Calcium acetate	99636	580	172	78
Ammonium sulfate	65144	226	288	51
DEAE-Cellulofine A-800				
P-1	16595	4.13	4018	13
P-2	6778	5.03	1348	5.3
Sephadex G-200 (1st)				
P-1	8804	1.45	6072	6.9
P-2	3837	2.15	1566	3.0
Sephadex G-200 (2nd)				
P-1	4905	0.57	8605	3.8
P-2	2948	0.98	3008	2.3

specific activities of the final purified materials were 8605 (*P*-1) and 3008 (*P*-2) representing purification factors of 269 and 94, respectively. The enzymes showed a broad single band by Disc-PAGE (Fig. 3) similar to those invertase [4] which is a glycoprotein and β-fructofuranosidase [9].

The molecular weight of the enzyme was determined by the Sephadex G-200 gel filtration method. As shown in Fig. 4, their molecular weight was estimated to be about 318000 (*P*-1) and 346000 (*P*-2). These values were similar to the one (340000) of β-fructofuranosidase from *A. niger* [8]. The sugar contents of *P*-1 and *P*-2 were 30% (w/w) and 53% (w/w), respectively. Sugar contents of *P*-1 and *P*-2 was higher than that of the *A. niger* enzyme.

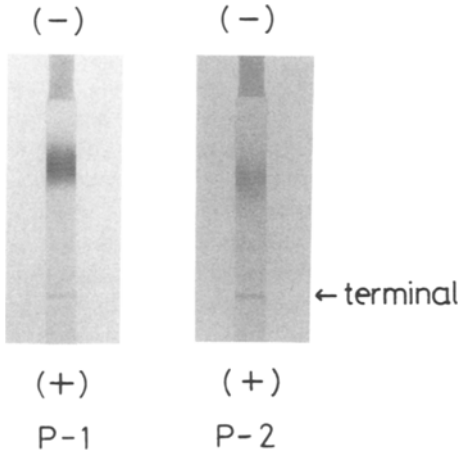

Fig. 3. Disc-PAGE of the enzymes from *Aureobasidium* sp. ATCC 20524 (60 μg protein).

Reaction profiles of the enzymes

Effects of pH on the reaction and stability of *P*-1 and *P*-2 are shown in Fig. 5. Optimum reaction pH of *P*-1 and *P*-2 were 4.5–5.5 and 4.5–6, respectively (Fig. 5A). The enzymes were stable at the range of pH 4–9 (Fig. 5B).

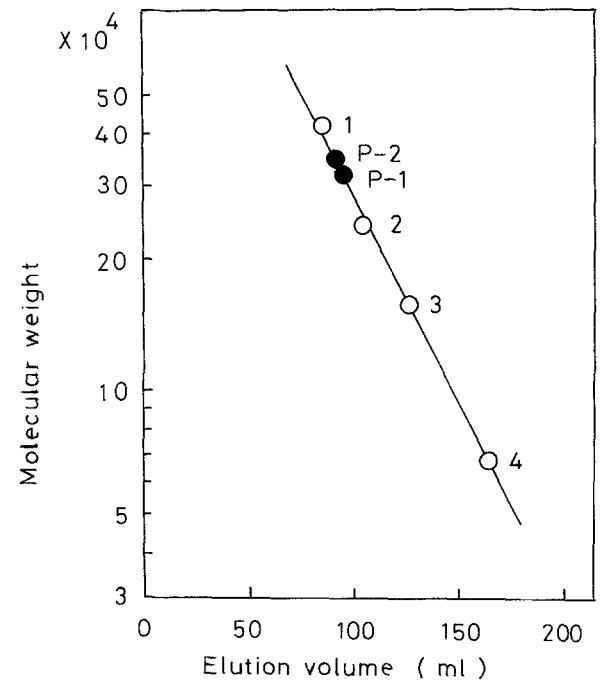

Fig. 4. Plot of logarithm of molecular weight of proteins against elution volume on Sephadex G-200. Standard proteins: 1 ferritin (450000); 2 catalase (240000); 3 aldolase (158000); 4 albumin (bovine serum; 68000).

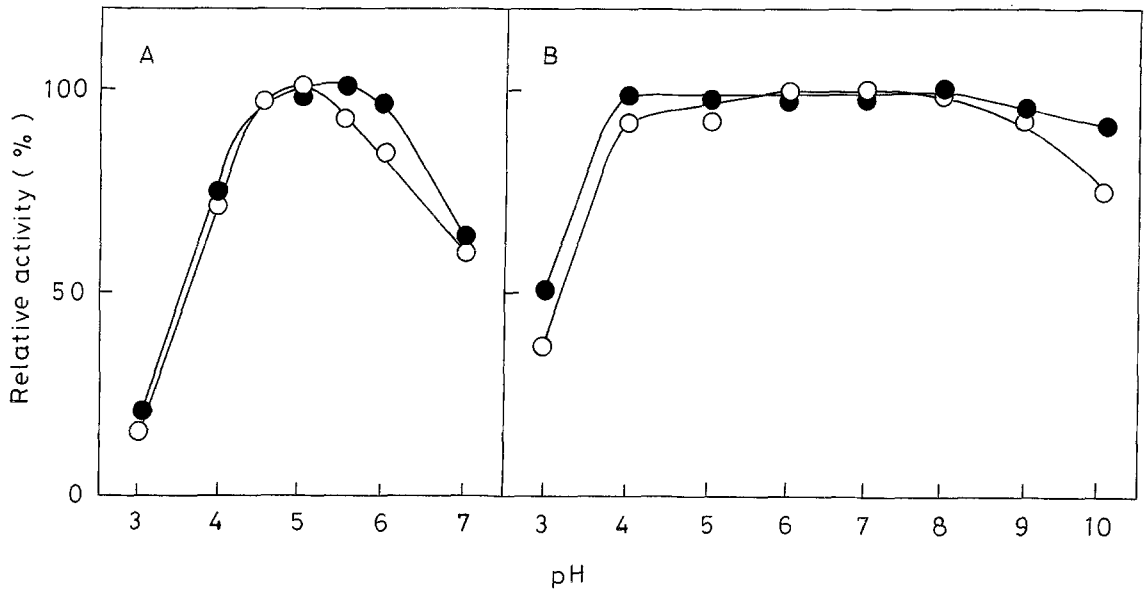

Fig. 5. Effect of pH on the activities and stabilities of the enzymes from *Aureobasidium* sp. ATCC 20524. Symbols: *P*-1, ○; *P*-2, ●. The activities were measured after 3 h incubation at each pH to determine stabilities of the enzymes.

Optimum pH of *P*-1 and *P*-2 were almost similar to the *A. niger* enzyme [8] and the *Penicillium* enzyme [9]. *P*-1 and *P*-2 were stable at a wide range of pH (like the *A. niger* enzyme) and more stable than the *Penicillium* enzyme.

Effects of temperature on the reaction and stability of *P*-1 and *P*-2 are shown in Fig. 6. Optimum reaction temperatures for both enzymes were 50–55 °C (Fig. 6A). *P*-1 and *P*-2 retained more than 94% and 98% activities at 50 °C but inactivated completely at 70 °C after 15 min (Fig. 6B).

Optimum temperature of *P*-1 and *P*-2 was similar to the *A. niger* enzyme but differed from the *Penicillium* enzyme. P-1 and P-2 were stable at higher temperatures than both the *A. niger* and *Penicillium* enzymes.

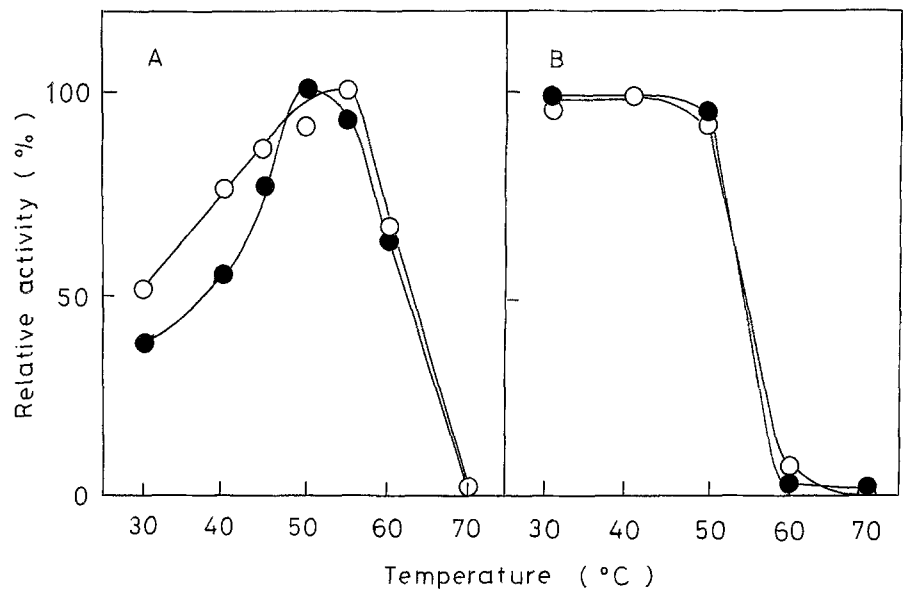

Fig. 6. Effect of temperature on the activities and stabilities of the enzymes from *Aureobasidium* sp. ATCC 20524. Symbols: *P*-1, ○; *P*-2, ●. The activities were measured after 15 min incubation at each temperature to determine stabilities of the enzymes.

In Fig. 7, the time course of enzymatic reaction by *P*-1 and *P*-2 at 50 °C is shown. *P*-1 and *P*-2 produced 1-kestose from sucrose and released glucose into the reaction mixture and had no sucrose-hydrolysing activity. The time course of enzymatic reaction by *P*-1 at 40 °C is also shown in Fig. 8. Fructose was released into the reaction mixture during extended time reaction as well as by the reaction of *P*-2.

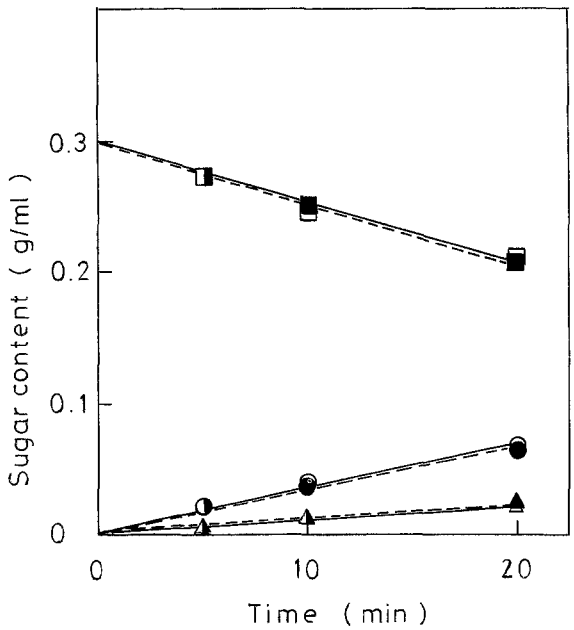

Fig. 7. Time course of the enzymatic reaction by the enzymes from *Aureobasidium* sp. ATCC 20524 at 50 °C. Symbols: 1-kestose, *P*-1, –○–; *P*-2, ··●··; glucose, *P*-1, –△–; *P*-2, ··▲··; sucrose, *P*-1, –□–; *P*-2, ··■··.

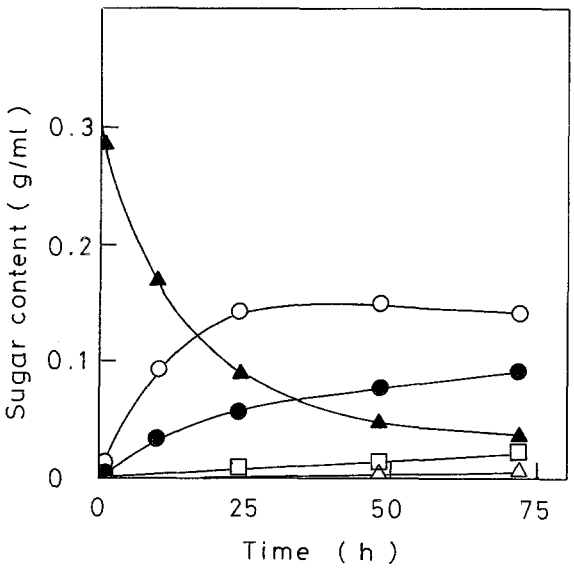

Fig. 8. Time course of the enzymatic reaction by *P*-1 at 40 °C. Symbols: 1-kestose, ○; glucose, ●; fructose, △; sucrose, ▲; other fructooligosaccharides, □.

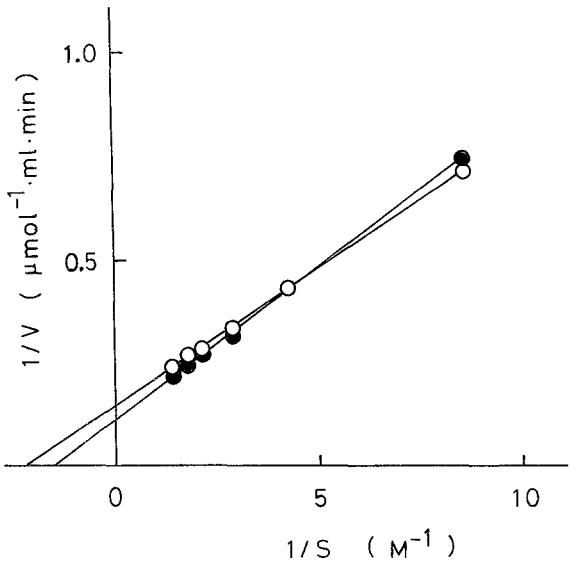

Fig. 9. Lineweaver-Burk plot of the enzymes from *Aureobasidium* sp. ATCC 20524 for the determination of K_m and V_{max} for sucrose. Symbols: *P*-1, ○; *P*-2, ●.

A Lineweaver-Burk plot of *P*-1 and *P*-2 is shown in Fig. 9. The K_m values for the sucrose of *P*-1 and *P*-2 were 0.47 M and 0.65 M, respectively. These values were higher than those for the *A. niger* enzyme (0.29 M). V_{max} for sucrose of *P*-1 and *P*-2 were 6.84 and 8.73 μmol/ml min, respectively.

Effect of metal ions and inhibitors on the reaction of enzymes

The activities of *P*-1 and *P*-2 were stimulated by Ba^{2+} and Ca^{2+} at concentrations of 1 mM. The profiles of effect of Ba^{2+}, Ni^{2+} and Fe^{3+} to *P*-1 and *P*-2 were very different than those from the *Penicillium* enzyme [9]. Ba^{2+} and Ni^{2+} decreased the activity of the *Penicillium* enzyme and increased that of *P*-1 and *P*-2. Fe^{3+} increased the activity of the *Penicillium* enzyme and decreased that of *P*-1 and *P*-2. *P*-1 and *P*-2 enzymes inhibited *p*-chloromercuribenzoate (PCMB) at concentrations of 1 mM.

These properties suggest that *P*-1 and *P*-2 are identical apart from their carbohydrate content. The enzyme profiles of *P*-1 and *P*-2 were considered to be different from both the *A. niger* enzyme [8] and the *Penicillium* enzyme [9].

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