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

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SUMMARY

Two extracellular β-fructofuranosidases (*E*-1 and *E*-2) from *Aureobasidium* sp. ATCC 20524, producing 1-kestose (1^F-β-fructofuranosyl-sucrose) from sucrose, were purified to homogeneity. Molecular weights of the enzymes were estimated to be about 304000 (*E*-1) and 315000 (*E*-2) Da by gel filtration. The enzymes contained 33% (w/w) (*E*-1) and 27% (w/w) (*E*-2) carbohydrate. The K_m values for sucrose of *E*-1 and *E*-2 were 0.34 and 0.28 M, respectively. The enzymatic profiles of these enzymes were almost identical to intracellular enzymes *P*-1 and *P*-2 except for the differences in carbohydrate content and K_m values of *E*-2 and *P*-2.

INTRODUCTION

In previous papers, we described the production of β-fructofuranosidases by *Aureobasidium* sp. ATCC 20524 [4] and the enzymatic profiles of these intracellular enzymes [5]. The enzymatic product, fructo-oligosaccharide (1-kestose), is very important because of its favorable functional properties for use in health food [6]. The characteristics of intracellular enzymes *P*-1 and *P*-2 were almost identical except for their carbohydrate content [5]. The strain also secreted a considerable amount of enzyme into culture broth after entering the stationary growth phase [4]. Extracellular enzyme is useful because it can be isolated by simple methods such as filtration or centrifugation, thus eliminating the need to recover it from cells. This investigation of the purification and properties of the extracellular enzymes was carried out to determine whether or not the extracellular enzymes were similar to the intracellular enzymes. Such a confirmatory study has not been done, as yet.

In the present paper, studies of the purification and properties of the extracellular enzymes were performed as described for the intracellular enzymes in an earlier publication to facilitate direct comparisons.

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MATERIALS AND METHODS

Cultivation of *Aureobasidium* sp. ATCC 20524 for enzyme production was carried out in liquid culture (sucrose 20%, yeast extract 2%, $NaNO_3$ 1%, K_2HPO_4 0.75%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 6.5–7) at 30 °C for 5 days as described previously [5]. Culture broth was centrifuged and the supernatant fluid was used for enzyme purification. Two volumes of ethanol were added to the crude enzyme solution. The resultant precipitate was collected and dissolved in 0.02 M Tris-HCl buffer (pH 8). To this solution, $(NH_4)_2SO_4$ was added to 100% saturation followed by centrifugation. The supernatant fluid was collected and dialysed against 0.02 M Tris-HCl buffer (pH 8). These procedures were carried out on ice. The dialysate was treated on a DEAE-Cellulofine A-800 column (0.9 × 30 cm) and then a Sephadex G-200 column (1.5 × 87 cm) as described previously [5]. Molecular weights of purified enzymes were estimated by Sephadex G-200 (1.5 × 87 cm) gel filtration according to the method of Andrews [1]. Disc-electrophoresis of the purified enzymes was performed as described previously [5] according to the method of Davis [2]. Enzyme activity was assayed in a reaction mixture consisting 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), at 50 °C for 20 min using HPLC and glucose oxidase methods as described previously [5]. Protein and carbohydrate were assayed by the method of Lowry et al. [7] and Dubois et al. [3], respectively.

RESULTS AND DISCUSSION

Purification of the enzymes

The results of purification of the extracellular β-fructofuranosidases from *Aureobasidium* sp. ATCC 20524 are shown in Table 1. The two β-fructofuranosidases (*E*-1 and *E*-2) were separated from each other by DEAE-Cellulofine A-800 ion exchange column chromatography corresponding to *P*-1 and *P*-2 of intracellular enzymes [5]. The specific activities (U/mg protein) of the final purified materials were 2531 (*E*-1) and 2216 (*E*-2) representing purification factors of 84 and 74, respectively. Each enzyme component showed a single band as did the intracellular enzymes by Disc-PAGE [5]. Their molecular weights were estimated to be about 304000 (*E*-1) and 315000 (*E*-2) Da by the Sephadex G-200 gel filtration. This compares to 318000 (*P*-1) and 346000 (*P*-2) for the intracellular enzymes [5]. Carbohydrate contents of *E*-1 and *E*-2 were 33 and 27% (w/w), respectively. This compares to 30% (*P*-1) and 53% (*P*-2) respectively [5].

Enzymatic profiles

The optimum reaction pH for *E*-1 and *E*-2 was 5–5.5 and the enzymes were stable in the range of pH 4–8, retaining 90% of their activity. The optimum reaction temperature for both enzymes was 50–55 °C. Both enzymes were stable at 50 °C but were almost completely inactivated at 70 °C after 15 min. These results were similar to those for the intracellular enzymes [5]. The production pattern for 1-kestose, glucose and fructose at 50 °C (Fig. 1) and 40 °C (Fig. 2) by *E*-1 and *E*-2 were identical to those of the intracellular enzymes [5]. The K_m values for sucrose of *E*-1 and *E*-2 by Lineweaver-Burk plots were 0.34 M and 0.28 M, respectively (Fig. 3). The V_{max} values for *E*-1 and *E*-2 were 6.66 and 8.70 μmol/ml/min, respectively. The K_m value of *E*-2 was lower than

TABLE 1

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

Step	Activity (U)	Protein (mg)	Specific activity (U/mg protein)	Yield (%)
Crude extract	166400	5472	30	100
Ethanol	104500	670	156	63
Ammonium sulfate	100500	309	326	60
DEAE-Cellulofine A-800				
E-1	15128	18.4	822	9.1
E-2	7675	12.9	595	4.6
Sephadex G-200 (1st)				
E-1	5858	3.2	1831	3.5
E-2	3527	2.4	1470	2.1
Sephadex G-200 (2nd)				
E-1	4303	1.7	2531	2.6
E-2	1821	0.5	2216	0.7

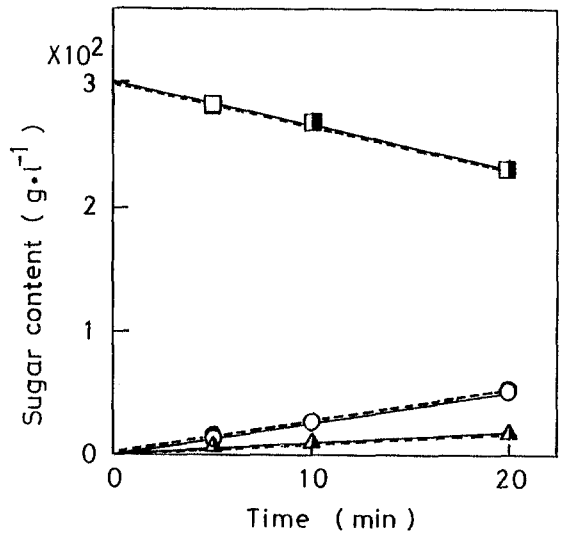

Fig. 1. Time course of the enzymatic reaction by the extracellular enzymes from *Aureobasidium* sp. ATCC 20524 at 50 °C. Symbols: 1-ketose, *E*-1, –○–, *E*-2, --●--; glucose, *E*-1, –△–, *E*-2, --▲--; sucrose, *E*-1, –□–, *E*-2, --■--.

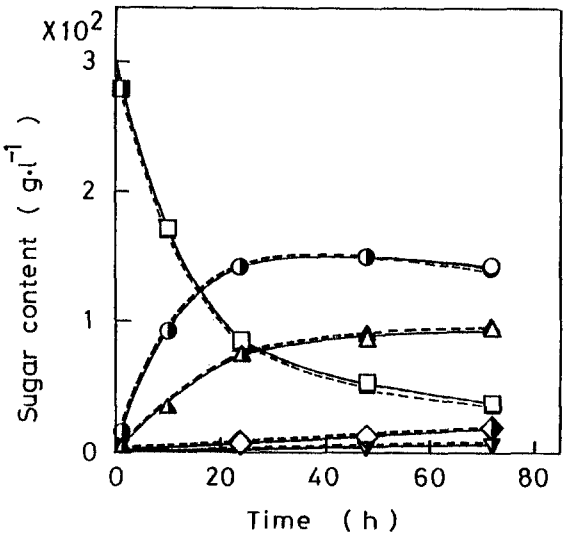

Fig. 2. Time course of the enzymatic reaction by the extracellular enzymes from *Aureobasidium* sp. ATCC 20524 at 40 °C. Symbols: 1-ketose, *E*-1, –○–, *E*-2, --●--; glucose, *E*-1, –△–, *E*-2, --▲--; fructose, *E*-1, –▽–, *E*-2, --▼--; sucrose, *E*-1, –□–, *E*-2, --■--; other fructo-oligosaccharides, *E*-1, –◇–, *E*-2, --◆--.

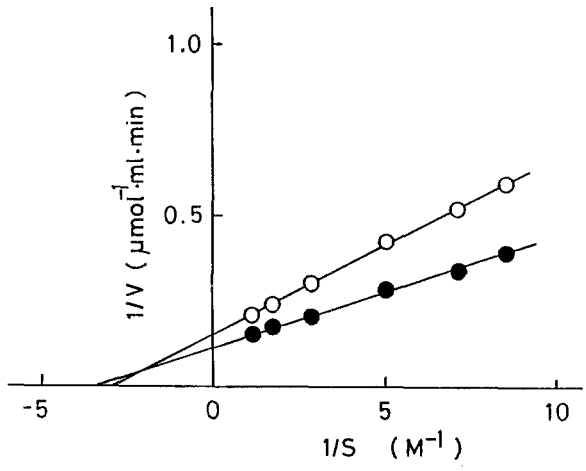

Fig. 3. Lineweaver-Burk plot of the extracellular enzymes from *Aureobasidium* sp. ATCC 20524 for the determination of K_m and V_{max} for sucrose. Symbols: *E*-1, –○–; *E*-2, –●–.

that of *P*-2 (0.65 M) [5]. It is considered that the affinity to substrate of *E*-2 increased because of the decrease in its carbohydrate content. The activities of *E*-1 and *E*-2 were inhibited by mercury (to 1.3 and 2.8%), copper (to 0.3 and 6.7%) and lead ions (to 2.0 and 15.4%) and *p*-chloromercuribenzoate (to 83.9 and 86.3%) at the concentration of 1 mM. The profiles of effects of metal ions and inhibitors were similar to those of the intracellular enzymes [5].

These studies suggest that the extracellular and intracellular enzymes from *Aureobasidium* sp. ATCC 20524 are similar except for the difference of carbohydrate content and K_m values between *E*-2 and *P*-2.

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