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Purification and Characterization of Invertase from *Aspergillus niger*

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Abstract. Invertase produced by a strain of *Aspergillus niger* showed the following main characteristics: maximum activity at 60°C, pH 5.0; K_m with sucrose as substrate, 0.0625 mM; V_{max} 0.013 mol/min; and free energy 9132 cal/mol. The metal ions and *p*-chloromercuribenzoate (PCMB) acted as inhibitors respectively.

Invertase (β-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26) catalyzes the cleavage of sucrose to glucose and fructose. Today, invertase is one of the most widely used enzymes in the food industry, especially in the preparation of jams and candies [8]. It has been extensively studied in yeasts and *Neurospora* sp. The enzyme is a glycoprotein, with mannose being the major component of the carbohydrate moiety [3, 11, 15]. The present paper reports the purification and characterization of invertase obtained from *Aspergillus niger*.

Materials and Methods

Microorganism. The strain of *A. niger* used throughout this work was isolated from mouldy lemons. It was maintained by monthly transfer to Czapek agar slants incubated at 30°C and stored at 4°C.

Fermentation medium. Czapek medium modified by Dox [1], containing 1 g/L sucrose, was used.

Inoculum. The fermentation medium was inoculated with 2.6×10^6 spores/ml from a stock culture and incubated for 18 h at 30°C in shaken flasks.

Enzyme production. Fermentation was carried out at 30°C in 500-ml shaken flasks containing 100 ml of fermentation medium and 10 ml of inoculum. After 48 h the mycelium was removed by filtration through SS595 filter paper and washed several times with distilled water. After adding 100 ml of 0.2 M acetate buffer, pH 4.5, plus 1.4 mM of 2-mercaptoethanol, the mycelial mixture was sonicated and centrifuged at 17,000 rpm for 20 min at 4°C. The supernatant was used as crude extract.

Enzyme purification. This process was performed at 4°C as follows: 285 ml of crude extract was brought to 45% ammonium sulfate saturation and the resulting precipitate removed by centrifugation at 17,000 rpm, 20 min. Ammonium sulfate was added to the supernatant up to 80% saturation. The precipitate was collected by centrifugation as above, dissolved in 2 ml of 0.2 M acetate buffer (pH 4.5) plus 1.4 mM of 2-mercaptoethanol and dialyzed overnight against the same buffer.

The dialyzed precipitate was applied to a column (3 × 60 cm) of Sephadex G-150 previously equilibrated with 0.2 M acetate buffer (pH 4.5) containing 1.4 mM of 2-mercaptoethanol and eluted at a rate of 30 ml/h with the same buffer. Fractions of 2 ml were collected, and fractions 43–49 were pooled. The total volume was applied to a column (1.5 × 10 cm) of DEAE Sephadex A-50. A linear NaCl gradient from 0 to 1 M in 200 ml of 0.2 M acetate buffer plus 1.4 mM of 2-mercaptoethanol was applied. Fractions of 2 ml were collected at a flow rate of 30 ml/h. As in the previous step, only the fractions with maximal invertase-specific activity (fractions 65–67) were pooled. This purified extract was dialyzed against the same buffer for 12 h and stored at 4°C. It was used for the determination of the main characteristics of the enzyme.

Protein estimation. Protein was measured by the method of Lowry et al. [9], with bovine serum albumin as a standard.

Enzyme assay. Enzymatic activity was determined at 30°C at pH 4.5, measuring the reducing sugars released with the Somogy-Nelson reagent [12, 14]. All of the reactions were made against enzyme and reagent as the blank. One unit of invertase was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of sucrose per ml per min at pH 4.5 and 30°C.

Kinetics constants. Michaelis-Menten constant (K_m) and Maximum velocity (V_{max}) were determined as a function of the sucrose concentration at 30°C and pH 4.5.

Effect of pH. The range of pH tested, from 2 to 8, was obtained by adding to the reaction mixture 0.2 M glycine-HCl buffer (pH 2–3), 0.2 M acetate buffer (pH 4–6) or 0.2 M phosphate buffer (pH 7–8).

Effect of temperature. It was tested with the reaction mixture at pH 4.5 in the range from 25°C to 75°C.

Effect of ions and PCMB. The effects of cations and anions were tested at 30°C and pH 4.5.

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Table 1. Purification of invertase from *Aspergillus niger*

Purification step	Total units	Protein (mg/ml)	Specific activity (units/ mg prot.)	Purification factor	Yield (%)
Crude extract	2.85	0.09	11.11	1	100
Ammonium sulfate 45–80%	42.5	0.45	18.88	1.69	14.90
Sephadex G-150	2.6	0.052	25	2.25	0.91
DEAE Sephadex A-50	2.4	0.025	96	8.65	0.84

Substrate specificity. The substrates used in the assay mixtures were: 0.2 M trehalose, melicitose, raffinose, and stachyose; 1% inulin; 0.02 M cellobiose, lactose, and maltose.

Estimation of the molecular weight. The molecular weight was estimated by gel filtration through Sephadex G-150. The column size was (3 × 60 cm), and the eluent 0.2 M acetate buffer (pH 4.5) plus 1.4 mM of 2-mercaptoethanol. The standard proteins were bovine serum albumin (67,000), ovoalbumin (45,000), alcalin phosphatase (100,000), and catalase (250,000).

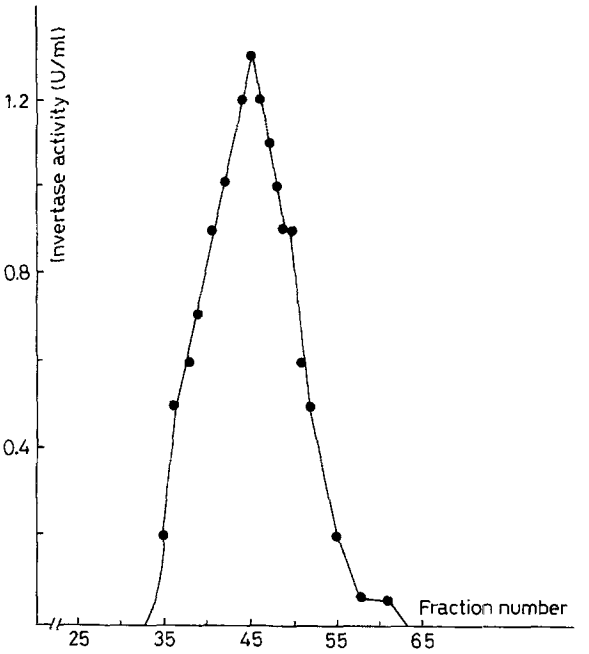

Fig. 1. *Aspergillus niger* β-fructofuranosidase purification step by Sephadex G-150 chromatography. Experimental conditions are described under Materials and Methods.

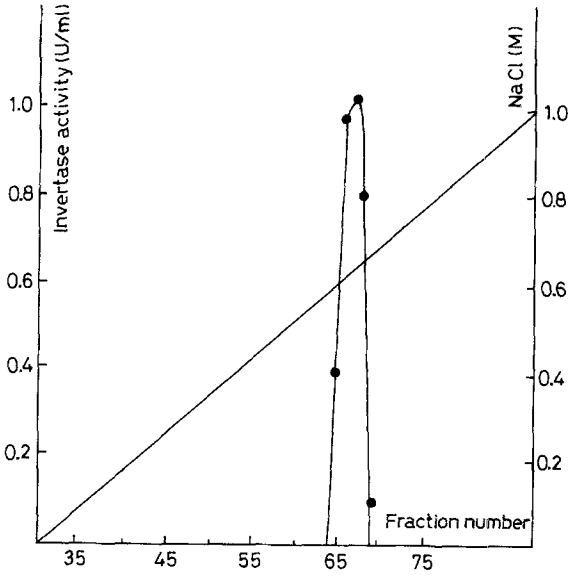

Fig. 2. *Aspergillus niger* β-fructofuranosidase purification step by DEAE Sephadex A-50 chromatography. Experimental conditions are described under Materials and Methods.

Results and Discussion

Enzyme purification. After the purification steps, the specific activity of the crude extract, expressed as units of invertase per mg protein dry weight, increased from 11.11 to 96, the yield of the purification procedure being about 0.84% (Table 1). The elution profiles from the gel filtration and chromatographic column showed a single peak with a symmetrical distribution of the activity (Figs. 1, 2).

Properties and characteristics of the enzyme

Kinetic constants. According to the results of the Lineweaver-Burk plot (Fig. 6), the values for K_m and V_{max} were 0.0625 mM sucrose and 0.013 mol/min respectively.

Effect of pH and temperature. Like other fungal invertases [5, 10], the activity occurred in the acid region, mainly in the pH range from 4 to 6 (Fig. 3), with a maximum at pH 5.0. Small variations from this value significantly impaired the activity. Concerning temperature, the maximum activity took place at 60°C (Fig. 4), the free energy, calculated from the Arrhenius plot, being 9132 cal/mol (Fig. 5).

Effect of metal ions. Since the invertases of yeasts and filamentous fungi might be inhibited by different kinds of ions [4, 7, 13], the effect of several ions at a concentration of 5 mM was tested. It was found that copper, cobalt, or magnesium reduced the activity by 50%; the same concentrations of potassium, sodium, calcium, nitrate, and sulfate by 30%; while phosphate and mercuric ions completely inhibited the activity of the enzyme.

Effect of p-chloromercuribenzoate (PCMB). In the presence of *p*-chloromercuribenzoate, 3.25 mM, the activity was reduced by 43%, and by 100% if the concentration was 6 mM. As PCMB is a specific

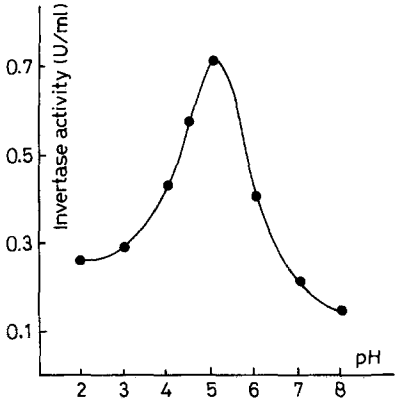

Fig. 3. Effect of pH on *Aspergillus niger* β-fructofuranosidase activity.

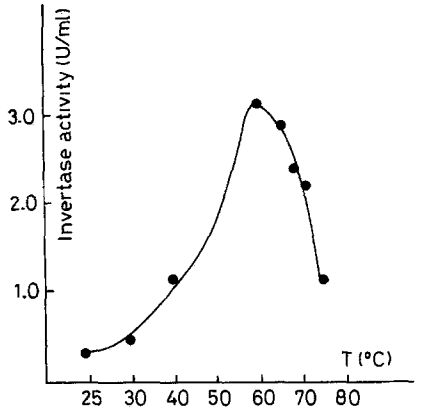

Fig. 4. Effect of temperature on *Aspergillus niger* β-fructofuranosidase activity.

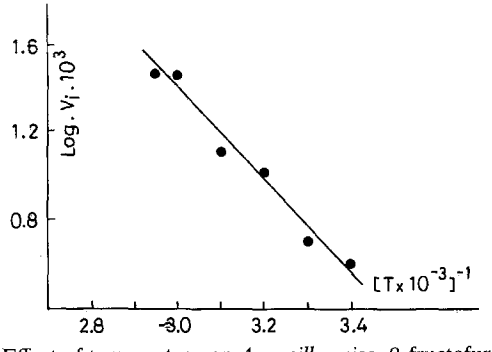

Fig. 5. Effect of temperature on *Aspergillus niger* β-fructofuranosidase activity (Arrhenius plot).

SH-blocking agent, these results suggest the involvement of sulfhydryl groups as well as a strong substrate protective effect.

Molecular weight. Gel filtration through Sephadex G-150 gave an estimated molecular weight of 95,000, while that of the simple protein band obtained with SDS-PAGE was 47,000, which suggests a dimeric

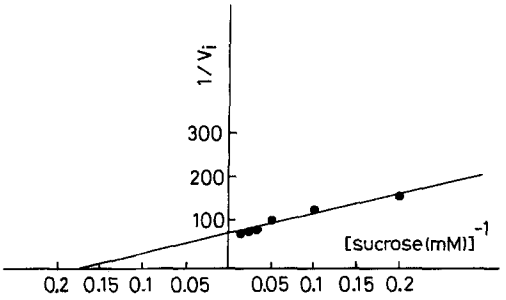

Fig. 6. *Aspergillus niger* β-fructofuranosidase, K_m and V_{max} determinations with different concentration of sucrose as substrate (Lineweaver-Burk plot).

structure like that reported for invertases from *A. ficuum* [2], and *Schwanniomyces occidentalis* [8], but different from that described for invertases from other *A. niger* and *A. japonicus* strains [4, 6].

Substrate specificity. The enzyme was able to hydrolyze sucrose, raffinose, and stachyose, but not maltose, lactose, cellobiose, melicitose, trehalose, and inulin. As sucrose is both a β-fructofuranoside and an α-glucoside, these results suggest that the enzyme is a β-fructofuranosidase, able to attack the sucrose molecule from the fructose end.

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