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Isolation and Identification of Trehalase from *Pullularia pullulans*

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Trehalase has been isolated from *Pullularia pullulans*. The enzyme, which is specific for trehalose, was purified approximately 800-fold. The optimal *p*H was found to be 4.0 and the Michaelis dissociation constant, K_m, was determined to be 3.2×10^{-3} M.

Trehalose has been found in microorganisms, vertebrates, invertebrates, and phanerogams (15). In fungi, trehalose functions as a reserve carbohydrate sustaining respiration and accelerating germination (6, 7, 14). Trehalose has recently been isolated from the yeastlike fungus *Pullularia pullulans* in which its accumulation parallels the increase of pigment formation in the cells (12, 13). *P. pullulans* is exceedingly common and found on fruits, vegetables, meat products, and cheese and is involved in the deterioration of paint and discoloration of lumber. Thus, it seemed of interest to undertake the isolation and characterization of trehalase from these cells.

P. pullulans (strain NRRL YB-4515) was grown in a medium consisting of (Difco) 0.3% malt and yeast extracts, 1.5% glucose, 0.0135% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% KH_2PO_4, and 0.02% NH_4Cl. After 5 days of shaking on a rotary shaker (220 rev/min and 2.5-inch thrust) at 28 C, the cells were harvested by centrifugation and washed first with 1% NaCl solution and then with distilled water until free from chloride. The cells were broken by adding to each gram of cells 3 g of 0.1-mm glass beads and 3 ml of 0.1 M sodium acetate buffer (*p*H 5.5) in a Sorvall Omni-mix. The rupturing was performed at 5 C at a rheostat setting of 100 for 1 hr, at which point microscopic examination indicated 80 to 90% breakage. The supernatant fluids were then centrifuged at 1,370 × *g* for 10 min, and the resulting residue was washed several times with 0.1 M acetate buffer (*p*H 5.5), hereafter referred to as "standard buffer." The washings were combined with the supernatant fluids, and the combined mixture was centrifuged at 6,590 × *g* for 10 min followed by centrifugation of the supernatant at 105,400 × *g* for 60 min.

Enzyme activity was measured by employing samples of the fractions mixed with 1 ml of sugar solution containing 10 μmoles of trehalose dissolved in standard buffer. After 15, 30, 45, and 60 min of incubation at 37 C, the amount of reducing sugar was determined by the Folin-Wu method (3). A unit of enzyme activity is defined as that amount of enzyme which produced 1.0 μmole of glucose per min per mg of protein at 37 C. Protein content of the enzyme solutions was determined by the procedure of Lowry et al. (11).

Ammonium sulfate was added to the supernatant liquid containing the enzyme with stirring at 5 C to a concentration of 40% saturation. After removal of the precipitate by centrifugation, the addition of ammonium sulfate was continued until a 70% saturation was obtained. After separation of the precipitate by centrifugation (20,000 × *g*), the supernatant was dialyzed against distilled water until free from ammonium sulfate. A small portion of this solution was dialyzed against standard buffer, whereas the remaining portion was lyophilized. The precipitates obtained from the 40 and 70% ammonium sulfate solutions were dissolved in small amounts of water and dialyzed against standard buffer. Samples of the lyophilized fraction were weighed out, dissolved, and along with samples of all solutions, tested for protein determination and enzyme activity. All trehalase activity was found in the 70% supernatant.

For the purification of the enzyme, columns (10 by 250 mm) packed with diethylaminoethyl cellulose (DEAE cellulose) were used. The DEAE cellulose was equilibrated in 0.005 M sodium phosphate buffer (9).

A 100-mg amount of the lyophilized material was dissolved in 2 ml of 0.01 M phosphate buffer (*p*H 7.75) and subjected to DEAE cellulose chromatography in a continuous decreasing *p*H gradient from *p*H 8.0 to 4.0 (9). The fraction collected between *p*H 4.0 and 5.0 contained most of

the enzyme activity. A summary of the purification is shown in Table 1. The procedure enabled approximately 800-fold purification of the enzyme with a recovery of 23% of the total enzyme activity.

The enzyme solutions tested produced a constant activity with time as well as a direct relationship between protein concentration and production of reducing sugar. The Michaelis constant of the purified enzyme for trehalose, calculated by the method of Lineweaver and Burk (10), was 3.2×10^{-3} M, which is about the order of magnitude reported for vertebrate and mammalian trehalases (2, 17) and trehalases from other sources (1, 4). The *p*H optimum of the enzyme was 4.0, appreciably lower than that of most trehalases reported to date (1, 4, 5, 7, 8, 17). How these related to the individual metabolic characteristics of the organism is a matter of further investigation.

The following carbohydrates, at 10 μmoles/ml, were tested as substrates for the purified trehalase: cellobiose, melibiose, gentiobiose, lactose, maltose, sucrose, raffinose, α-methyl-D-glucoside, and β-methyl-D-glucoside. No detectable increase in reducing sugar (as glucose) was observed (<0.03 μmole per min per mg of protein) from the nonreducing carbohydrates, and <0.3 μmole per min per mg of protein was detected from the reducing sugars. The level of specific activity of the partially purified enzyme is some 15-fold greater than that obtained by Roberts and Tovey (16) from *Selaginella martensii* and 4-fold greater than that obtained by Hill and Sussman with *Neurospora crassa* (6).

TABLE 1. *Recovery of enzyme activity from ruptured cells of Pullularia pullulans*

Fractions	Vol recovered (ml)	Protein (μg/ml)	Total enzyme units recovered	Specific activity (μmoles of glucose per min per mg of protein)
1,370 × *g*	200	14,120	6,800	0.08
6,590 × *g*	190	14,000	6,000	0.08
105,400 × *g*	160	12,100	3,260	0.06
40% $(NH_4)_2SO_4$ precipitate	80	500	40.0	0.033
70% $(NH_4)_2SO_4$ precipitate	20	175	2.8	0.026
70% $(NH_4)_2SO_4$ supernatant	150	200	3,000	3.3
DEAE cellulose	80	10	1,600	66.6

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