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Partial Purification and Properties of a Trehalase from *Streptomyces hygroscopicus*

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The enzyme α,α'-glucoside 1-glucohydrolase, which catalyzes the hydrolysis of trehalose, was isolated from *Streptomyces hygroscopicus* and was purified approximately 80-fold. The enzyme was completely specific for trehalose as substrate. None of the other naturally occurring glucose disaccharides exhibited any significant activity. The *p*H optimum for enzymatic activity was found to be 6.5 and the K_m was estimated to be approximately 1.8×10^{-2} M. The product of the reaction was identified as D-glucose by chemical, chromatographic, and enzymatic methods. The presence of this enzyme was demonstrated in several species of *Streptomyces* and related organisms.

Trehalose is found in many organisms, such as yeasts, insects, fungi, and bacteria, where it functions as a reserve carbohydrate. Trehalase activity has been detected in most of these organisms. An excellent review of trehaloses and trehalases has been made by Birch (4). More recently, trehalose synthesis (21) and metabolism (5) in the cellular slime mold *Dictyostelium discoideum* have been described.

As reported in a previous communication (6), an enzyme which catalyzes the transfer of glucose from guanosine diphosphate-D-glucose (GDPG) to glucose-6-phosphate has been isolated from *Streptomyces hygroscopicus*. The purification and properties of this new enzyme have been reported in another communication (*in preparation*). This enzyme was also detected in a number of other *Streptomyces* species (7). In each of these organisms, it was shown that the vegetative mycelia contained readily detectable amounts of trehalose, which was characterized by its physical properties following crystallization.

The present report deals with the purification and properties of a specific trehalase (α,α'-glucoside 1-glucohydrolase) from *S. hygroscopicus* as well as with the demonstration of trehalase activity in a number of other *Streptomyces* species.

MATERIALS AND METHODS

Materials. All chemicals were obtained from commercial sources unless otherwise indicated. A lyophilized culture of *Leuconostoc mesenteroides* NRRL B-1424, which produces a dextran from which kojibiose and isomaltose were isolated, was kindly supplied by Allene R. Jeanes of the U.S. Department of Agriculture. The culture was maintained as previously described (12). Kojibiose and isomaltose were isolated by acetolysis (25). Nigerose was obtained by hydrolysis of nigeran (3) and laminaribiose from hydrolysis of laminarin (20). Sophorose was a gift from H. G. Fletcher of the National Institutes of Health. Calcium phosphate gel was made by the method of Keilin and Hartree (14) or was purchased from the Sigma Chemical Co., St. Louis, Mo. Hydroxylapatite, made by the method of Miyazawa and Thomas (16), was kindly donated by E. Bresnick, Baylor University College of Medicine.

Analytical methods. Glucose was determined by either the reducing sugar method of Nelson (18) or with glucose oxidase obtained from Worthington Biochemical Corp., Freehold, N.J. (11). Hexose was determined by the anthrone method (15) and protein by the method of Sutherland et al. (24).

Paper chromatography was performed with either Whatman no. 1 or no. 3MM filter paper. Chromatographic solvents were solvent I, propan-1-ol-ethyl acetate-water (7:1:2); solvent II, butan-1-ol-acetic acid-water (8:1:1); solvent III, butan-1-ol-pyridine-water (6:4:3). Sugars were detected on chromatograms by use of the alkaline silver nitrate reagent (26).

Culture conditions. S. hygroscopicus was grown as previously described (6). After 8 days of growth at room temperature on a New Brunswick rotary shaker, cells were harvested by filtration on a Büchner funnel and washed several times with ice-cold distilled water; the cell paste was stored at −10 C until used.

Assay of enzyme. For routine assay, incubation mixtures contained the following components, in a final volume of 0.1 ml: sodium cacodylate buffer

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(*p*H 6.5), 20 μmoles; trehalose, 3.0 μmoles; and an appropriate amount of enzyme. The mixtures were incubated at 37 C for 15 min. The reaction was stopped by heating the tube for 10 min in a boiling-water bath. The release of glucose was measured either by the Nelson method or by glucose oxidase. Both methods gave comparable results.

One unit of enzymatic activity is defined as that amount of enzyme which hydrolyzes 1 μmole of trehalose in 1 min at 37 C.

Purification of the trehalase. Lysozyme was used to disrupt the cells (27). A 60-g amount of cell paste was suspended in 180 ml of 0.1 M potassium phosphate buffer (*p*H 7.5), containing 0.5 mg of ethylenediaminetetraacetic acid per ml and 1 mg of egg-white lysozyme (Sigma Chemical Co.) per ml. After incubation at room temperature for 1 hr, the suspension was centrifuged at 30,000 × *g* for 10 min. The supernatant fluid contained the enzymatic activity. All steps were conducted at 0 C unless otherwise indicated.

Manganese precipitation and first ammonium sulfate fractionation. To 180 ml of crude extract, 7.2 ml of 1 M $MnCl_2$ was added slowly with stirring. The mixture was allowed to stand for 5 min and then was centrifuged. The precipitate was discarded, and 31.5 g of solid ammonium sulfate (30% saturation) was added to the supernatant liquid. After centrifugation, an additional 38.7 g of solid ammonium sulfate was added to the supernatant liquid (60% saturation). After centrifugation, the precipitate was dissolved in 30 ml of distilled water and was dialyzed overnight against distilled water.

Calcium phosphate gel treatment. To 33 ml of the ammonium sulfate fraction, 49.5 ml of calcium phosphate gel (15 mg/ml) was added. The mixture was allowed to stand for 15 min and then was centrifuged. The supernatant fluid was discarded and the gel was resuspended in 50 ml of 0.04 M potassium phosphate buffer, *p*H 6.5. The gel suspension was stirred mechanically for 30 min and then was centrifuged. The enzymatic activity was released from the gel and was recovered in the supernatant fluid.

Second ammonium sulfate fractionation. To 50 ml of the supernatant liquid from the above treatment, 12.1 g of solid ammonium sulfate (40% saturation) was added. The precipitate was removed by centrifugation and discarded, and an additional 5.1 g of solid ammonium sulfate (55% saturation) was added to the supernatant liquid. The precipitate was removed by centrifugation, dissolved in 10 ml of distilled water, and dialyzed overnight against distilled water.

Hydroxylapatite chromatography. A 10-ml amount of the second ammonium sulfate fraction was applied to a column (1.5 × 6 cm) of hydroxylapatite, which had previously been equilibrated with 0.001 M potassium phosphate buffer (*p*H 6.5). The column was washed with 50 ml of 0.001 M potassium phosphate buffer, *p*H 6.5. The enzyme was eluted stepwise with 50-ml portions of 0.01 M, 0.02 M, and 0.03 M potassium phosphate buffer (*p*H 6.5). Enzymatic activity was removed at 0.03 M. With this procedure, the enzyme was purified approximately 80-fold with 10% recovery (Table 1). This enzyme fraction was used in all of the following experiments.

The enzyme was found to be stable to freezing at all stages of purification. The purified enzyme showed no loss in activity after storage at −20 C for 4 weeks and could be frozen and thawed several times with no significant loss of activity.

RESULTS

Effect of time, enzyme concentration, and substrate concentration. The hydrolysis of trehalose was linear with respect to time and enzyme concentration (Fig. 1). The rate of the reaction was proportional to trehalose concentration to 1.2×10^{-2} M, and the K_m was estimated to be 1.8×10^{-2} M (Fig. 2).

Effect of pH. Figure 3 illustrates the effect of *p*H on the rate of hydrolysis of trehalose. The optimal *p*H in cacodylate buffer was found to be approximately 6.5. Potassium phosphate buffer at the concentration used in the standard assay procedure (0.2 M) caused approximately 20% inhibition of enzymatic activity. Tris(hydroxymethyl)aminomethane buffer at the same concentration completely inhibited enzymatic activity.

Stoichiometry of the reaction. Incubation of trehalose with the purified enzyme resulted in the formation of 2 moles of glucose for each mole of trehalose consumed.

Characterization of the product. As would be expected, the product of the reaction was easily identifiable as glucose. Approximately 12 μmoles of the product was isolated, by paper chromatography in solvent I, from a large incubation mixture. On development of the chromatogram with alkaline silver nitrate, only two spots were observed, corresponding to glucose and trehalose. The area corresponding to glucose was cut out and eluted. On further chromatography of this region in two other solvents, it was again found to have the same mobility as authentic D-glucose (Table 2). In addition, samples tested for reducing sugar, total hexose, and activity with D-

TABLE 1. *Purification of trehalase*

Fraction	Total units	Specific activity (units/mg of protein)
Crude extract	35.0	2.2×10^{-2}
Manganous chloride + ammonium sulfate	30.0	7.2×10^{-2}
Calcium phosphate gel	28.3	14.8×10^{-2}
Ammonium sulfate	15.8	21.8×10^{-2}
Hydroxylapatite	3.3	166.7×10^{-2}

glucose oxidase gave a ratio of hexose to reducing sugar to glucose oxidase-positive substance of 1.02:1:0.93.

Substrate specificity. The purified enzyme was found to be completely specific for trehalose (Table 3). None of the other naturally occurring glucose disaccharides exhibited any significant activity. No α- or β-glucosidase activity was detectable when the enzyme was tested with the α- or β-*p*-nitrophenyl glucosides. The enzyme did, however, exhibit a slight activity towards trehalosamine.

Detection of trehalase activity in Streptomyces other than S. hygroscopicus. Table 4 shows the specific activities of trehalase in various species of *Streptomyces* and also in two other actinomycetes. It can be seen that, to a greater or lesser extent, the enzyme is present in all of the species thus far examined. It should be noted, however, that although the relative specific activities differed as much as 100-fold, all of the values were significant.

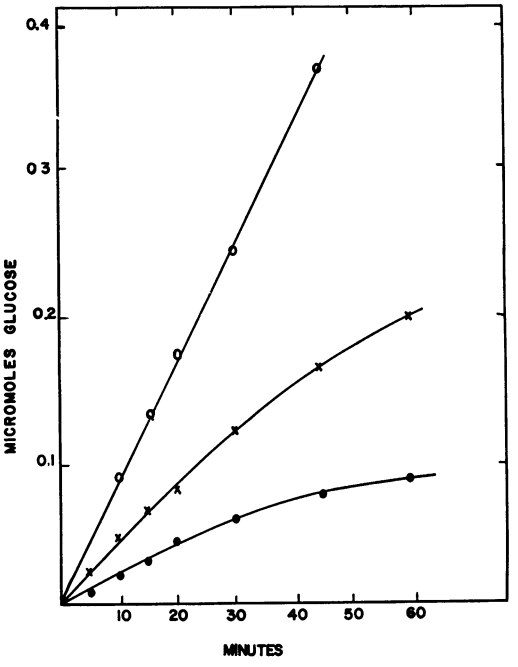

FIG. 1. *Effect of time and enzyme concentration on rate of hydrolysis of trehalose. Incubation mixtures contained, in a final volume of 1 ml, sodium cacodylate (pH 6.5), 200 μmoles; trehalose, 30 μmoles; and trehalase, 1.34 × 10⁻² units (●), 2.62 × 10⁻² units (×), and 5.33 × 10⁻² units (○). At the times indicated, 0.1-ml samples were withdrawn and analyzed for reducing sugar formation.*

TABLE 2. *Paper chromatographic identification of the product formed from trehalose*[a]

Compound	R_g[a]		
	I	II	III
Glucose	1.00	1.00	1.00
Galactose	0.92	0.94	0.91
Mannose	1.40	1.26	1.10
Unknown	1.00	1.00	1.00

[a] Ratio to glucose in solvents I, II, and III.

TABLE 3. *Substrate specificity of trehalase*[a]

Disaccharide	Linkage	Amt of glucose formed per hr
		μmoles
Trehalose	α1 → 1	0.72
Kojibiose	α1 → 2	0.00
Sophorose	β1 → 2	0.00
Nigerose	α1 → 3	0.00
Laminaribiose	β1 → 3	0.00
Maltose	α1 → 4	0.04
Cellobiose	β1 → 4	0.00
Isomaltose	α1 → 6	0.03
Gentiobiose	β1 → 6	0.00
Trehalosamine	α1 → 1	0.10

[a] Incubation mixtures contained, in a final volume of 0.1 ml, sodium cacodylate (*p*H 6.5), 20 μmoles; enzyme, 6.67 × 10⁻² units; and 3 μmoles of disaccharide. Mixtures were incubated for 15 min, and glucose formation was measured by glucose oxidase.

TABLE 4. *Distribution of trehalase*[a]

Organism	Specific activity (units/mg of protein)
Streptomyces hygroscopicus	1.55 × 10²
S. lavendulae	0.12 × 10²
S. rimosus	2.25 × 10²
S. aureofaciens	0.1 × 10²
S. antibioticus	0.07 × 10²
S. griseus	0.18 × 10²
S. venezuelae	0.05 × 10²
S. virginiae	0.1 × 10²
Micromonospora chalcae	0.02 × 10²
Mycobacterium smegmatis	0.03 × 10²

[a] Incubation mixtures contained, in a final volume of 0.2 ml, sodium cacodylate (*p*H 6.5), 40 μmoles; trehalose, 6 μmoles; and an appropriate amount of the dialyzed crude extract of the organism indicated. Mixtures were incubated for 15 min, and the glucose formed was measured by the Nelson method for reducing sugar.

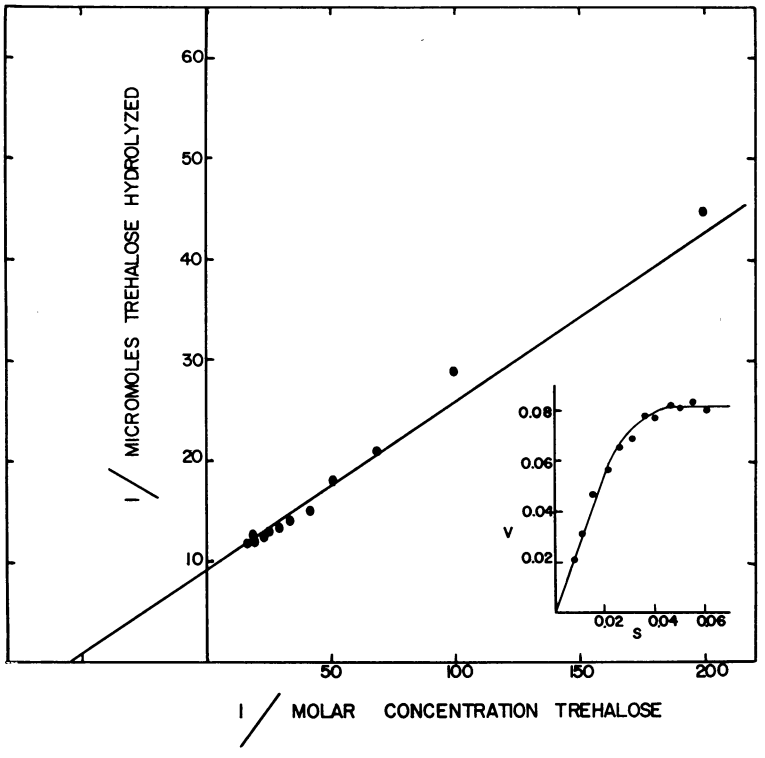

FIG. 2. *Effect of substrate concentration on trehalase activity. Incubation mixtures contained, in a final volume of 0.2 ml, 20 μmoles of cacodylate buffer (pH 6.5); 0.67 × 10^{-2} units of enzyme; and trehalose, in various amounts. The mixtures were incubated for 15 min, and glucose formation was measured by the reducing sugar method.*

DISCUSSION

The trehalase isolated from *S. hygroscopicus* is markedly similar to that isolated from the hybrid yeast by Avigad et al. (2). The yeast enzyme was shown to have a *p*H optimum of 6.9 and a K_m of 10^{-2} M, which are comparable with the *p*H optimum of 6.5 and K_m of 1.8×10^{-2} M of the *S. hygroscopicus* enzyme. These values are in marked contrast to those found in other organisms, such as yeasts (17, 19), insects (8, 22), and *Neurospora* (10).

Trehalose has been found in large quantities (15% of the total carbohydrate) in *Neurospora* spores (23). A trehalase has been crystallized from the mycelia (10), and it has been shown that, although trehalase activity is present in spores, there is a great increase in the amount of enzyme upon germination (9).

Similarly, trehalose is also present in high concentration in the spores of the cellular slime mold *D. discoideum*. On germination, trehalase activity increases markedly. Trehalose is rapidly hydrolyzed and the glucose formed is further metabolized (5).

Very low levels of trehalase activity have been found in the spores of *S. hygroscopicus*. The level of this enzyme in germinating spores has not yet been determined. In addition, the levels of trehalose present during germination have not been examined. However, as trehalose appears to be a reserve carbohydrate in many organisms, this may be its function in *Streptomyces*.

It is interesting to note that trehalosamine inhibits the germination of the spores of *D. discoideum* (5). Trehalosamine has also been found to exert an antibacterial effect against mycobacteria (1). In the present study on the trehalase from *S. hygroscopicus*, preliminary evidence indicates that trehalosamine can act not only as a substrate for trehalase but also as an inhibitor of trehalase when trehalose is used as substrate. Presumably, therefore, trehalosamine acts as a competitive inhibitor.

An enzyme which catalyzes the cleavage of trehalose phosphate to trehalose and inorganic phosphate has also been detected in crude extracts of *S. hygroscopicus*, but the specificity of

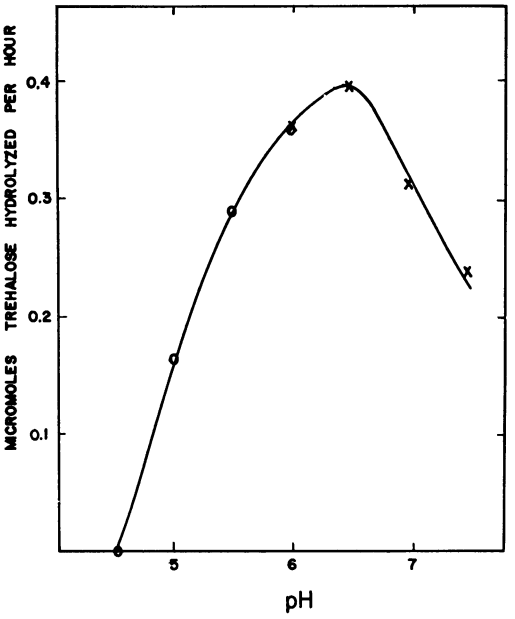

FIG. 3. *Effect of pH on trehalase activity. Incubation mixtures were as described in the text. Acetate buffer from pH 4.5 to 6 (○) and cacodylate buffer from pH 6 to 7.5 (×) were used.*

this phosphatase has not been determined (A. E. Hey and A. D. Elbein, *unpublished data*).

Trehalase activity has been detected in all of the other species of *Streptomyces* tested thus far. Although the specific activities differed by as much as 100 times, there was a significant level of activity in each species. It would be of interest to measure the relative changes in the levels of the enzyme through the vegetative phase of growth, as well as in the spores of each species before and during germination. With this information and with more information on the properties and relative levels of the other enzymes in the system, such as GDPG pyrophosphorylase, trehalose synthetase, and trehalose phosphate phosphatase, a more thorough understanding of the role of trehalose in *Streptomyces* can be achieved.

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