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Activity and Heat Stability of Trehalase from the Mycelium and Ascospores of *Neurospora*

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Trehalases from the ascospores of *Neurospora tetrasperma* and the mycelium of *N. crassa* were compared. Enzymes from both sources have identical electrophoretic mobilities, K_m's, responses to pH, immunological reactions, and activities in low-molarity buffers. Because both enzymes are so similar, conclusions about the properties of the ascospore enzyme may be made by studying mycelial trehalase. Mycelial trehalase is most active and stable in low-molarity buffers. The enzyme exists in at least three species; the smallest has a molecular weight between 105,000 and 125,000 and is predominant in low-molarity buffers at 37 C. The stability of trehalase to heating at 65 C can be increased by increasing enzyme concentration and by the addition of polyols. Ascospores contain large amounts of trehalose, which protects trehalase from heat inactivation at 65 C. The importance of this phenomenon in vivo and its relationship to the localization of trehalase in ascospores is discussed.

Trehalase (EC 3.2.1.28, α,α'-glucoside 1-glucohydrolase) and its substrate trehalase are present throughout most of the life cycle of *Neurospora* (9, 11, 30). Mycelial trehalase has been purified and partially characterized (10), and trehalose has been isolated from ascospores and crystallized (30).

Large amounts of trehalose accumulate in conidia (9) and ascospores (30), even though trehalase is present as well. Although dormant ascospores do have the ability to metabolize exogenous glucose (2), endogenous trehalose is not metabolized until activation of ascospores by a heat shock (60 C for 5 to 30 min) or furfural treatment (29), which result in the immediate utilization of trehalose, presumably mediated by trehalase (30).

Yu et al. (35) have shown that trehalase is more stable to temperatures of 60 or 65 C in intact ascospores than in extracts. Extracts whose small molecules have been removed by dialysis are much less stable to heating than are nondialyzed extracts. If the dialysate is concentrated and added back to dialyzed trehalase, not only is the enzyme protected, but it is activated about 30 to 40% in the first hour of heating at 65 C. These small molecules from ascospores were able to protect trehalase obtained from either the mycelium or conidia of both *Neurospora crassa* and *N. tetrasperma*.

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It has been assumed that trehalase from the ascospores of *N. tetrasperma* and from the mycelium of *N. crassa* are identical (35) in order to study the properties of the mycelial enzyme and relate them to the behavior of trehalase in ascospores. Because it is much easier to obtain large quantities of trehalase from mycelium as compared with ascospores, this approach also has been followed in this paper. However, it was possible to obtain enough trehalase from ascospores for limited comparisons with the mycelial enzyme to test the assumption that trehalases from both sources are identical.

The experiments reported in this paper will deal with the following subjects: (i) comparison of trehalase obtained from ascospores of *N. tetrasperma* and the mycelium of *N. crassa*; (ii) investigation of the conditions under which mycelial trehalase is most active and stable; and (iii) a further investigation of the ability of ascospore extracts to protect trehalase from heat inactivation.

The report is taken in part from a dissertation submitted by L.I.H. to the University of Michigan, Ann Arbor, in partial fulfillment of the requirement for the Ph.D. degree.

MATERIALS AND METHODS

Purification of mycelial trehalase. Strain 89601 A of *N. crassa* was grown for 6 days at 20 C and harvested according to the method of Hill and Suss-

man (10). The mycelium was then lyophilized and broken into fine pieces in a Waring blender and ground for 5 min with a minimal amount of 0.05 M phosphate buffer, pH 5.6. The resulting suspension was further homogenized in a Gifford-Wood Minimill (10) without sand. The procedures used differed from previous ones (10) in that the enzyme in the 60 to 100% ammonium sulfate fraction was used instead of that in the 0 to 80% fraction. Purified trehalase (trehalase stock solution) was stored at −20 C at a protein concentration of approximately 0.25 mg/ml in 0.05 M phosphate buffer, pH 5.6.

Purification of ascospore trehalase. The ascospores used were between 3 and 4 years old. They had been obtained by crossing strains 374.4 and 374.5 of *N. tetrasperma* on a medium containing 4.0% potato-dextrose agar, 0.5% yeast extract (Difco) after 5 weeks at 20 C (18). Spores were removed from storage at 4 C, washed in 0.8% ethylenediaminetetraacetic acid and then in deionized water, and ground in a mechanically driven syringe (7). Broken ascospores were centrifuged to remove the spore walls. The supernatant fluid was vacuum dialyzed at 0 C to concentrate the spore proteins (35). Equal amounts of the dialyzed proteins and 0.05 M phosphate buffer, pH 5.6, were mixed, and the resulting solution was processed as was the mycelial extract (10).

Assays. Protein was estimated by the method of Lowry et al. (18) or by spectrophotometry (14).

Trehalase was assayed as previously described (10). Reducing sugars produced by trehalase activity were measured by the method of Nelson (23). The frozen stock solution was diluted 1,000 fold for assays of purified mycelial trehalase. Spore trehalase was diluted either 500 or 250 fold for assay.

Glucose was assayed enzymatically with glucose oxidase. (Glucostat assay kits were obtained from Worthington Biochemical Corp., Freehold, N.J.)

Acrylamide gels were prepared by the methods of Eilers et al. (6).

Sephadex column chromatography. The state of aggregation and approximate molecular weight of trehalase were determined with a Sephadex G-150 column. The column (2 by 30 cm) was run at 4 to 6 C and had a flow rate ranging from 24 to 28 ml/h, depending upon the molarity of buffer used. Samples were applied in 2-ml portions and the eluates from columns were collected in 3-ml fractions.

Heat-inactivation studies. The following procedure was used for testing the effect of buffers with molarities less than or equal to 0.05 M. Trehalase stock solution was diluted 1,000 fold and heated in an Erlenmeyer flask. At appropriate intervals samples were withdrawn and cooled in an ice bath.

The effect of buffers with molarities of 0.05 M or higher was tested with trehalase stock solutions diluted 10-fold in buffer and placed in small (10 by 75 mm) test tubes and heated. At appropriate intervals samples were withdrawn and diluted 100-fold with cold buffer so that the final molarity was 0.05 M and the pH as close as possible to 5.6. The diluted enzyme was then assayed for trehalase activity.

In the above two procedures, after dilution to the concentration used for assay, half of each sample of enzyme was dialyzed for 2 h against 1 liter of buffer (the molarity to be used in the assay) and then assayed for trehalase activity.

When the effects of nonionic solutes on the heat stability of trehalase were tested, the trehalase stock solution was diluted 10-fold in the solution to be tested and heated in a small test tube. At intervals, samples were withdrawn, diluted 100-fold with cold 0.0005 M acetate buffer, pH 5.4, and assayed for trehalase activity.

Tests of the effects of spore extracts and related materials were carried out on trehalase that was diluted 5-fold in the solution to be tested and heated in a small test tube. Samples were withdrawn and diluted 200 fold with cold 0.0005 M buffer for assay.

Ascospore extracts. Ascospores were processed as for the purification of trehalase, except that after vacuum dialysis the protein-free dialysate was kept (35). The dialysate was concentrated by using a rotary evaporator at 65 C.

To prevent enzyme action during extraction and dialysis, ascospores were boiled in 80% ethanol for 5 min. All grinding was carried out in 80% ethanol, and the spore extract was heated at 60 C to evaporate any remaining ethanol. The remaining solution was then vacuum dialyzed and concentrated as above.

The concentrated spore extract (1 ml obtained from 1 g [fresh weight] of ascospores) was then passed through a Dowex 50W-X8 column (200–400 mesh H^+ form) which was eluted with an amount of de-ionized water equivalent to four column volumes. The eluate was reconcentrated by rotary evaporation and passed through a Dowex 1-X8 column (200–400 mesh, formate form) which also was eluted with four column volumes of deionized water. The resultant neutral fraction was then brought to the starting concentration. Basic and acidic fractions were obtained by passing four column volumes of 4 N NH_4OH and 6 N formic acid through the above Dowex 50W and Dowex 1 columns, respectively, after the removal of the neutral materials.

The neutral fraction thus obtained was analyzed for glucose by glucose oxidase. Trehalose was analyzed by digestion of the neutral fraction with purified trehalase in order to form glucose, which was analyzed as above.

The content of the neutral fraction also was investigated by descending paper chromatography by using the following solvent systems: (i) *n*-propanol, ethyl acetate, and water (7:1:2) on Whatman no. 1 filter paper for 18 h; (ii) 2-butanone, acetic acid, and water saturated with boric acid (9:1:1) on Whatman no. 1 filter paper run twice for 6 h (17); and (iii) 2-butanone, acetone, water, and pyridine (150:30:20:1) on Whatman 3MM filter paper for 30 h (25) or on Whatman no. 1 filter paper for 60 h and rechromatographed for 18 h. The chromatograms were developed with the following spray reagents: (i) ammoniacal-silver nitrate (32); (ii) periodate-benzidine; and (iii) aniline-diphenylamine (1).

The neutral fraction was freed of both trehalose and glucose by incubation with purified trehalase followed by digestion with glucose oxidase (Type V, obtained from Sigma Chemical Co., St. Louis, Mo.). The resultant solution was gently boiled for 5 min to remove enzymes and accumulated hydrogen peroxide

and was then passed through Dowex columns as described above to remove gluconic acid and buffer ions. The composition of the neutral fraction from such digested extracts was analyzed with glucose oxidase as well as with descending paper chromatography with solvent system (ii) and spray reagent (ii).

The conductivity (reciprocal resistance) of the original spore extract was tested against known concentrations of sodium acetate by using a Wheatstone bridge.

RESULTS

Comparison of mycelial and ascospore trehalases. Trehalase from the mycelium of *N. crassa* strain 89601 A was purified about 680-fold and had a specific activity of 294,000 μg of glucose per h per mg of protein. Its activity was directly proportional to enzyme concentration in the range of 0.05 μg to 1.25 μg of protein/ml.

The enzyme from ascospores was purified 22-fold or an equivalent of 50-fold compared with crude mycelial extracts. The purification sequence is shown in Table 1. Unfortunately, during purification on diethylaminoethyl (DEAE)-cellulose, much of the enzyme activity was lost without much purification. This may have been due to the small amount used during these procedures.

Acrylamide gel electrophoresis shows that purified mycelial trehalase has only one band of trehalase activity (Fig. 1); this corresponds to one protein band (which sometimes appears as a doublet). There is one other protein band of consequence which appears only in the most purified trehalase fractions but has no associated trehalase activity. This band migrated more slowly than did the main trehalase band and may have been due to inactive subunits or aggregates of the enzyme. This view is supported by the detection of at least two different species of trehalase on a Sephadex G-150 column (see below). Both trehalases were identical by electrophoresis on polyacrylamide gels when stained for trehalase activity (Fig. 1). This is not surprising in view of the results obtained by Yu et al. (34), which show that most standard and exotic strains of *N. crassa* tested have identical trehalase bands which run in this position.

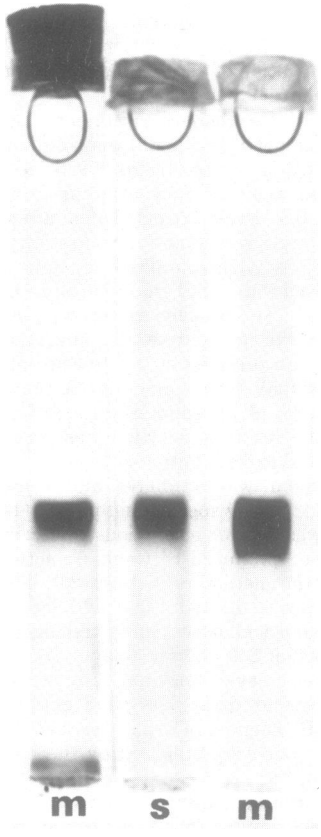

FIG. 1. *Acrylamide disk gels of ascospore (s) and mycelial (m) trehalases stained for trehalase activity. The gels on the right and center were run at the same time, and the gel on the left is from a separate experiment.*

TABLE 1. *Purification of trehalase from Neurospora tetrasperma ascospores*

Fraction	Total activity (μg of glucose/h)	Total protein (mg)	Sp act (μg of glucose per h/mg of protein)	Purification factor	Yield (%)
Crude extract	93,600	95.2	981	1.0 ×	100
Supernatant after 15 min at 60 C	95,160	54.1	1,760	1.8 ×	100 +
Supernatant after $MnSO_4$-NaOH removal of nucleic acids	101,760	31.8	3,200	3.3 ×	100 +
60 to 100% $(NH_4)_2SO_4$ cut	85,050	10.8	7,875	8.0 ×	90.9
After first DEAE-cellulose column	38,710	3.8	10,214	10.4 ×	41.4
After second DEAE-cellulose column	6,340	0.3	21,420	21.7 ×	6.8

Both trehalases had K_m's of approximately 1.5×10^{-4} M (Fig. 2), and the pH optima of the spore and mycelial enzymes in 0.05 M phosphate buffer were also very similar (Fig. 3). The pH optimum for trehalase from ascospores was 5.8, whereas that for the mycelial enzyme was 5.6. The pH versus activity curves differed by about the same amount on the acid side of the optima, but the two curves were almost identical on the basic side. This may perhaps be accounted for by the greater impurity of ascospore trehalase which, when added to 1 ml of 0.05 M phosphate buffer, pH 5.6, lowered the pH by 0.02 to 0.04 units. Mycelial enzyme caused no detectable change.

Mycelial and ascospore trehalases showed lines of identity when run on Ouchterlony plates with antibodies made against trehalase obtained from the mycelium of *N. crassa* strain 89601 A.

Ascospore trehalase showed about a 10% increase in activity in low-molarity buffers compared with its activity in 0.05 M buffer. This behavior was similar to that of the mycelial enzyme (see below).

Effect of ionic strength on the activity of mycelial trehalase. Increasing the molarity of acetate buffer decreases the activity of trehalase (Fig. 4). The lowest concentration of buffer used was 0.0005 M, and the activity of trehalase in 0.05 M buffer is taken as 100%. This concentration of buffer was set as a standard because it is the one previously used in assaying trehalase (10, 11, A. S. Sussman et al., Ind. J. Bot., in press). Moreover, enzyme activity in this buffer is fairly stable and varies little from assay to assay, therefore the results of different experiments can be related. Solutions (0.05 M) of $MgCl_2$, $MgSO_4$, $CaNO_3$, $CaCl_2$, or $(NH_4)_2SO_4$ in 0.05 M acetate buffer, pH 5.6, decreased trehalase activity an additional 5 to 15%. Therefore the decrease in trehalase activity caused by increasing ionic strength (Fig. 4) is characteristic of salts of both monovalent and divalent cations.

Decrease in enzyme activity due to increased buffer concentrations is not characteristic of acetate buffer alone, because sodium phosphate and NaOH-KH phthalate buffers give similar results. A series of experiments using various buffers is illustrated in Fig. 5, where trehalase activity is plotted as a function of the logarithm

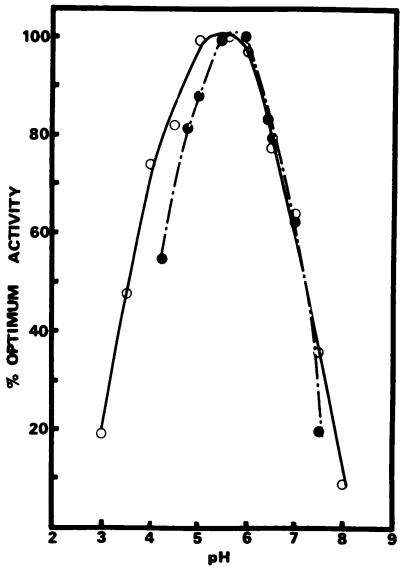

FIG. 3. *pH versus activity curves for mycelial* (○) *and ascospore trehalases* (●) *in 0.05 M phosphate buffer. For each enzyme its pH optimum is 100% activity.*

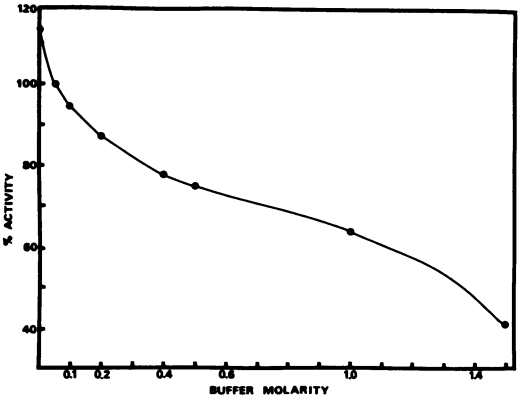

FIG. 4. *Percentage of trehalase activity versus molarity of acetate buffer, pH 5.6. Activity of trehalase in 0.05 M buffer is 100% activity. The lowest molarity tested was 0.0005 M.*

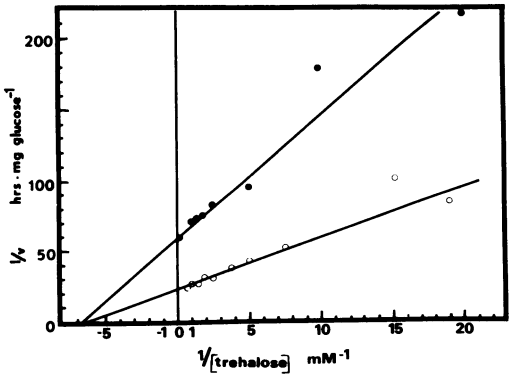

FIG. 2. *Lineweaver-Burke plots of mycelial* (○) *and ascospore* (●) *trehalases.*

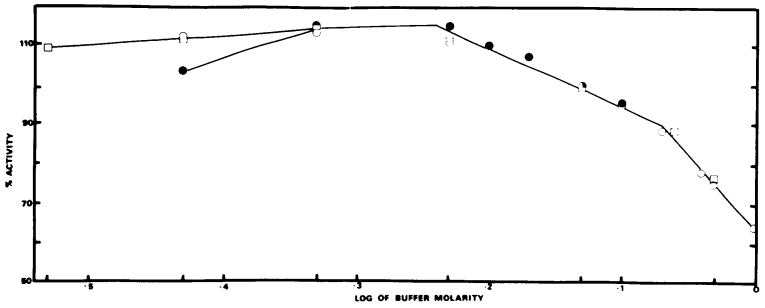

FIG. 5. *Percentage of trehalase activity versus logarithm of buffer molarity. Activity in 0.05 M buffer is 100% activity. Sodium phosphate buffer (pH 5.6), ●; sodium acetate buffer (pH 5.6), ○; potassium-hydrogen phthalate-NaOH buffer (pH 5.6), □.*

of buffer molarity. These results show that: (i) trehalase is most active at lower molarities ranging between 0.0005 and 0.005 M; (ii) trehalase activity decreases linearly with respect to the logarithm of buffer molarity from 0.005 to 0.2 M; and (iii) there is a sharp break in the slope at 0.2 M, after which the slope of the curve decreases at about twice the previous rate until 1.0 M. The relatively sharp changes in slope in Fig. 5 may indicate that trehalase undergoes some structural change in response to changing ionic strength.

Effect of ionic strength on the molecular weight of trehalase. A Sephadex G-150 column was used to test the hypothesis that changes in ionic strength cause a change in the molecular structure of trehalase (Fig. 6). Trehalase placed in 0.0005 M acetate buffer, pH 5.4, and kept at 0 C before application to the column eluted in two peaks; the first was in the void volume and therefore greater than 400,000 mol wt, and the second peak was equivalent to a mol wt between 160,000 and 250,000. When, instead, trehalase was placed in 1.0 M acetate buffer (pH 5.6) and kept cold before application, only one peak in the void volume was seen. However, a case may be made for the existence of some lower-molecular-weight species because of the long tail of this curve. When trehalase was heated at 37 C (assay temperature) for 30 min before application to the column, the following results were obtained. Trehalase in 0.0005 M buffer shifted to a molecular weight of approximately 105,000 to 125,000 with a shoulder, possibly representing higher-molecular-weight species. Buffer (1.0 M) induced a shift from the large species to the one of 160,000 to 250,000 mol wt, with a shoulder of lower molecular weight.

Effect of dialysis on trehalase activity. When trehalase was diluted to assay concentration in an appropriate buffer and then dialyzed against the same buffer at 0 C (5 ml of diluted trehalase solution against 1 liter of buffer), enzyme activity increased about 10 to 20% in 2 to 3 h (Fig. 7). When the top line (dialyzed for 2 h before assay) is compared with the line immediately below it (same treatment, but undialyzed), the increase in trehalase activity is clearly shown. The absolute amount of increase of activity is fairly constant regardless of the

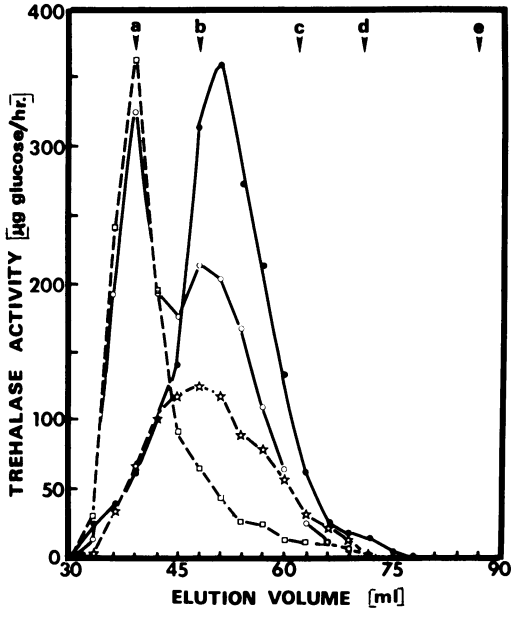

FIG. 6. *Elution profiles from a Sephadex G-150 column (2 by 30 cm). Trehalase in 0.0005 M acetate buffer (—○—) and 1.0 M acetate buffer (- -□- -) kept at 0 to 4 C before application to the column; trehalase in 0.0005 M acetate buffer (—●—) and 1.0 M acetate buffer (- -☆- -) heated at 37 C for 30 min before application to the column. Molecular weight markers: a, blue dextran (2,000,000); b, catalase (250,000) and γ-globulin (160,000); c, bovine serum albumin (67,000); d, ovalbumin (45,000); e, α-chymotrypsinogen (25,000).*

amount of heat inactivation the enzyme has undergone before dialysis.

Effect of ionic strength on the heat stability of trehalase. Both the molarity of the buffer and the concentration of enzyme affect the stability of trehalase (Fig. 7 and 8). The stock solution of trehalase was diluted either 10- or 1,000-fold for heat inactivation (see Materials and Methods). In Fig. 7, 100% activity is equivalent to the activity of trehalase in 0.05 M acetate buffer, pH 5.6. This graph takes into account the different initial activities of trehalase in different molarity buffers. All samples heated in buffer greater in concentration than 0.05 M were diluted to 0.05 M for assay. However, enzyme placed in 4.5 M buffer regained only 70% of its original activity upon dilution even without heating. Inactivation of

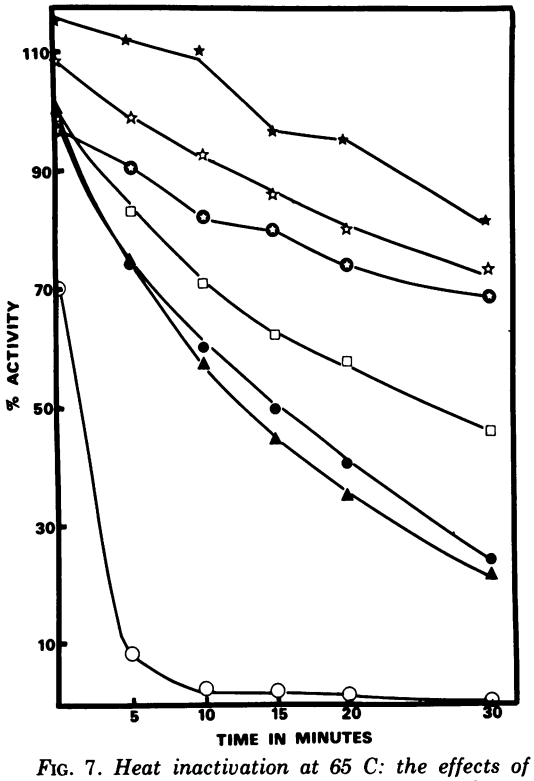

FIG. 7. *Heat inactivation at 65 C: the effects of buffer molarity and enzyme concentration. Shows percentage of trehalase activity versus minutes of heating at 65 C. Activity of trehalase in 0.05 M acetate buffer, pH 5.6, is 100% activity. Trehalase was diluted 1,000 fold in the following pH 5.6 acetate buffers: 5 × 10⁻⁶ M, ✪; 5 × 10⁻⁴ M, ☆; 5 × 10⁻² M, ●, for heat inactivation and assay. ★, enzyme heated in 5 × 10⁻⁴ M buffer and dialyzed against this same buffer for 2 h before being assayed. Trehalase was diluted 10-fold for heat inactivation in the following buffers: 5 × 10⁻² M, □; 0.9 M, ▲; 4.5 M, ○. Assays were done after 1,000-fold dilution to a final molarity of 0.05 M.*

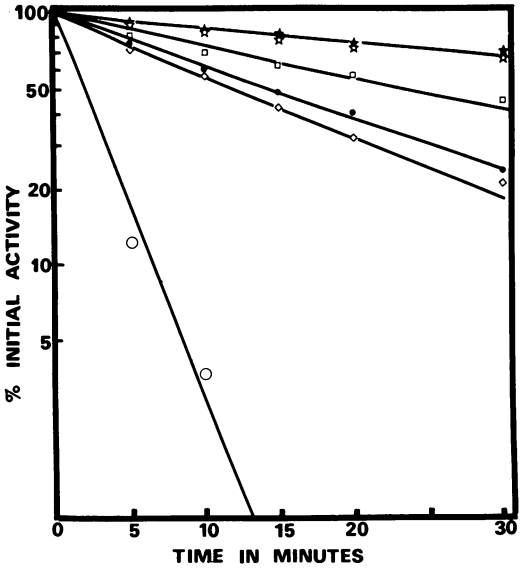

FIG. 8. *Heat inactivation at 65 C: the effects of buffer molarity and enzyme concentration. Shows Log of percentage of initial trehalase activity before heating versus minutes of heating at 65 C. See Fig. 7 for details. Symbols for 1,000-fold dilution: 5 × 10⁻⁶ M, ★; 5 × 10⁻⁴ M, ☆; 5 × 10⁻²M, ●. Symbols for 10-fold dilution: 5 × 10⁻² M, □; 0.9 M, ◇; 4.5 M, ○.*

trehalase in these ionic solutions is a first-order reaction (Fig. 8). In this graph, 100% activity is equivalent to the activity of the enzyme in various buffer systems before it is heated.

Therefore, trehalase is most active and stable in low-molarity buffers. An enzyme concentration difference of 100-fold (10-fold versus 1,000-fold) results in approximately a doubling of the half-life in the more concentrated solution (compare enzyme heated in 0.05 M buffer at 10-fold and 1,000-fold dilutions in Fig. 8).

Effect of nonionic substances on the activity of trehalase. Figure 9 shows the effects of increasing concentrations of glycerol, dimethyl sulfoxide (DMSO), ethanol, acetone, urea, and mannitol, which were used in 0.0005 M acetate buffer, pH 5.3 to 5.4. Experimentation was limited by the solubility of mannitol and inositol in water, and compounds such as DMSO and urea could not be buffered beyond a concentration of 2 M. (The last point on the urea curve has a pH of almost 8.) Lower concentrations of mannitol and inositol inhibited trehalase more than did similar concentrations of other compounds. However, mannitol and inositol did not appear to be effective competitive inhibitors, because they had to be used in high concentrations to achieve any significant inhibition. (The mannitol and inositol curves are almost identical, so only the mannitol curve is

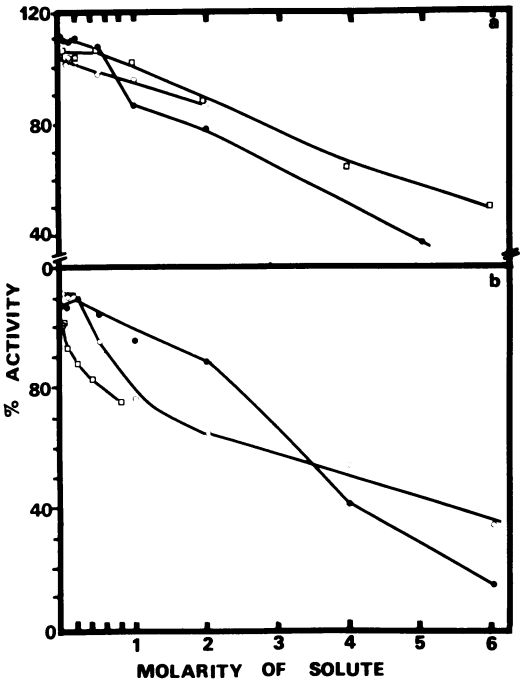

FIG. 9. *Percentage of trehalase activity versus molarity of nonionic solute in 0.0005 M acetate buffer, pH 5.4. Activity of trehalase in 0.05 M acetate buffer, pH 5.6, is 100% activity. Symbols (a): glycerol, ●; dimethyl sulfoxide, ○; ethanol, □. (b): urea, ●; acetone, ○, mannitol, □.*

shown.) The decrease in trehalase activity in the presence of compounds such as urea and ethanol was not due to any permanent inactivation caused by these compounds. Assays in either 2 M urea or 6 M ethanol were linear for at least 1 h, and full trehalase activity could be regained by diluting any of the concentrated solutions if they had been kept at 0 C.

Effect of nonionic compounds on the heat stability of trehalase. Heat-inactivation analyses were performed with trehalase stock solution diluted 10-fold in solutions of nonionic compounds. High concentrations of glycerol (5 and 9 M) protected trehalase from heat inactivation, whereas low concentrations were without effect (Table 2). Protection against heat inactivation was obtained also with concentrated solutions of sugar alcohols, including mannitol and inositol (near their limits of solubility), but low concentrations of these compounds had little or no protective ability. Glucose and trehalose, which also are polyols, were similarly effective stabilizing agents (Table 3). Low concentrations of ethanol and acetone did not change the stability of trehalase, whereas high concentrations inactivated the enzyme at 65 C (Table 2). Acetone and ethanol (each 5 M) apparently had no denaturing effect upon trehalase at 0 C. DMSO behaved differently in that high concentrations of this compound had no effect on trehalase, but low concentrations inactivated it.

Analysis of ascospore extracts. Following the techniques of Yu et al. (35), ascospores were extracted in deionized water. The extract was concentrated so that 1 ml of the extract corresponded to 1 g (fresh weight) of ascospores. Only the neutral fraction obtained from this extract increased the heat stability of trehalase. Both the acidic and basic fractions labilized trehalase even though the pH of these solutions was adjusted to 5.6 with small amounts of either 0.05 M sodium acetate or 0.05 M acetic acid.

The water-extracted neutral fraction contained 2.6% trehalose, 2.3% glucose, and about 0.1% mannitol (estimated from paper chromatography). However, ascospores of *Neurospora* are reported to contain little, if any, glucose (2, 30, F. I. Eilers, Ph.D. thesis, Univ. of Michigan, Ann Arbor, 1968). In order to avoid the enzymatic breakdown of spore constituents, ascospores were first boiled for 5 min in 80% ethanol before extraction was begun. The neutral fraction from this extraction procedure was concentrated as above and found to contain 5.4% trehalose, 0.07% glucose, and approximately 0.1% mannitol.

TABLE 2. *Heat inactivation of mycelial trehalase at 65 C in nonionic solutes*[a]

Compound	Molarity	Initial activity remaining after 30 min at 65 C (%)	Half-life (min)
Acetate buffer	0.0005	56	33
Glycerol	0.05	56	
	5.0	97	765
	9.0	97	
Ethanol	0.05	65	
	5.0	0	1
Inositol	0.05	66	
	0.5	80	
Mannitol	0.05	58	
	0.8	92	224
Acetone	0.05	56	
	5.0	0	1
Dimethyl sulfoxide	0.05	0	2
	5.0	56	

[a] All solutions were buffered in 0.0005 M acetate buffer, pH 5.3 to 5.4.

TABLE 3. *Heat inactivation of mycelial trehalase at 65 C*[a]

Solution	Initial activity remaining after 30 min at 65 C (%)
Control	24
Neutral fraction from the ethanol extract of ascospores	82
Neutral fraction after sequential digestion by trehalase and glucose oxidase	35
0.4% Trehalose (0.01 M)	48
4.0% Trehalose (0.1 M)	78
16.0% Trehalose (0.42 M)	85
4.0% Glucose (0.2 M)	65
14.0% Trehalose in 0.4 M acetate buffer, pH 6.5	70

[a] Measured as the effect of ascopore extracts and sugar solutions on the heat stability of trehalase.

These data indicate that if there is any protective factor in ascospores, it is probably trehalose or some undetected compound. To test this hypothesis, trehalase was heated in the presence of the neutral fraction of the ethanol extract and in known concentrations of trehalose (Table 3). The trehalose solutions used in these experiments were de-ionized by passage through Dowex columns so they would be equivalent in this way to the neutral fraction.

Trehalose did protect trehalase from heat inactivation, but not very effectively; low concentrations offered only slight protection and higher concentrations of trehalose did protect, but not completely. The neutral fraction of the ethanol extract offered about the same amount of protection as did a 4.0% trehalose solution. (The amount of trehalose hydrolyzed by trehalase during heating at 65 C is negligible compared to the total present and should not have influence these results.)

The neutral fraction from the ethanol extract was digested sequentially with trehalase and glucose oxidase. The digested neutral fraction now contained less than 0.004% glucose and 0.004% trehalose, plus mannitol and gluconic acid (about 0.1% as estimated from paper chromatography). Because the trehalose-free extract no longer increased the heat stability of trehalase significantly (Table 3), the protective factor in ascospore extracts probably was trehalose.

Ascospores washed and air-dried on a Büchner funnel contained 40% water. Therefore, it can be estimated that the internal solution of this batch of ascospores contained a minimum of 13.5% trehalose, if it is assumed that all of the water present resided in the cytoplasm. The internal solution of these ascospores had an ionic strength equivalent to a 0.4 M sodium acetate solution with a pH of 6.5. A solution containing 14% trehalose and buffered at pH 6.5 with a 0.4 M sodium acetate buffer offered less protection against heat inactivation than did a 4% trehalose solution (Table 3). The activity of trehalase in such a solution was only about 40% of its maximal activity.

DISCUSSION

Although the small amount of enzyme obtained from ascospores limited the number of criteria which could be compared, it is reasonable to assume that mycelial enzyme from *N. crassa* is very similar if not identical to the trehalase obtained from ascospores of *N. tetrasperma*. An objection to this may be made because the low yield of trehalase (6.8%) obtained may not be representative of all the trehalase present in ascospores. However, during the purification of mycelial (10) and ascospore enzymes, similar specific activities were obtained after one passage through DEAE-cellulose. Great losses of activity occur during chromatography on DEAE-cellulose in both mycelial (10) and ascospore preparations. Therefore, the low yield of ascospore trehalase probably was a result of the small amount of starting material, and the information gained by working with the mycelial enzyme will be assumed to hold for the ascospore enzyme as well.

The activity and heat stability of trehalase were studied in vitro in the hope of gaining some insights into the properties of trehalase and how it functions in ascospores. This is similar to the approach taken by Sadoff (27), who points out that this type of study has the advantage of distinguishing between the stability of isolated protein molecules and the viability of spores.

It appears as though low temperatures and high ionic strengths keep trehalase in a high-molecular-weight aggregate, whereas high temperatures (37 C) and low ionic strength favor dissociation into two species, a basic unit with a molecular weight of 105,000 to 125,000 and another species which may be a dimer or an unfolded variant of the basic unit. It cannot be determined from the data given in this paper whether all of the species of trehalase detected are enzymatically active, because the assay conditions used (30 min at 37 C) would dissociate any form of trehalase into the basic unit.

The greater stability of trehalase in buffers of low ionic strength can be correlated with the presence of the 105,000- to 125,000-mol wt species, whereas high ionic strengths would favor the aggregated and presumably, less stable species. Paradoxically, increasing the concentration of a trehalase solution results in increased heat stability. A possible explanation for this behavior would be that higher protein concentrations may prevent or retard the 105,000- to 125,000-mol wt species from dissociating into smaller subunits, which are more heat labile.

Other proteins exist whose state of aggregation can be altered by changing the ionic strength of the solutions in which they are placed (5, 12, 25, 26, 33). There are also several cases where high salt concentrations will either stabilize (19, 27, 28) or labilize (22) enzymes; changes in protein stability can be correlated with aggregation (19) or dissociation into monomers (21, 27, 28). The work on *Neurospora* invertase (22) is interesting because this invertase, like trehalase, is a sugar hydrolase existing in cell walls throughout much of the life cycle of this organism (3, 4, 11, 21, Sussman et al., in press). High salt (1 M NaCl) concentrations inactivate invertase, this being correlated with the dissociation of the polymeric form into subunits (22).

Dilution can cause the dissociation of aspartate transcarbamylase (15) and horse heart lactic dehydrogenase (20). In the latter case, dissociation is thought to be followed by changes in the tertiary conformation of subunits, thereby leading to inactivation. Perhaps the increase of trehalase activity during dialysis can similarly be related to changes in its state of aggregation.

None of the nonionic compounds tested enhanced the activity of trehalase over that displayed in low-ionic-strength buffers. Increasing the molarity of all the nonionic compounds tested (Fig. 9) inhibited trehalase, but not very drastically. Because increasing concentrations of all the compounds tested resulted in a similar slope of decreasing trehalase activity, similar means of inhibition for all compounds is suggested.

Glycerol was tested for its ability to stabilize trehalase because of its wide use as a stabilizing agent (13, 31). Unlike glycogen phosphorylase (8) and 17β-hydroxysteroid dehydrogenase (13, 31), trehalase is stabilized by polyols but not by high concentrations of other organic compounds like DMSO and simple alcohols, etc.

Ascospore extracts. The data in this paper indicate that trehalose is the compound in ascospore extracts that protects trehalase from heat inactivation. When trehalase is heated with trehalose at 65 C, hydrolysis occurs, resulting in glucose being formed. This increase in background sugar must be taken into account when trehalase is assayed after various intervals of heating. Previously, this was not considered because the composition of the ascospore extracts was unknown (35), therefore the activation and much of the protection of trehalase by concentrated ascospore extracts reported by Yu et al. (35) are artifacts. However, trehalose does protect trehalase from heat inactivation to a considerable extent.

The maximum activity of trehalase (assuming most of the spore trehalase was extracted and fully active) isolated from the group of spores used in these experiments is capable of digesting 80 to 90% of the trehalose found in these spores in the first 6 h of germination. This is equivalent to the percentage of trehalose digested by intact germinating ascospores as shown by Lingappa and Sussman (17). However, when ascospore trehalase is in a cytoplasm with a pH of 6.5 and an ionic strength equivalent to 0.4 M sodium acetate, it can function at only 40% of its maximal activity. This would not be sufficient to maintain the rates of trehalose utilization known to occur (17).

The data in this paper indicate that trehalose can protect trehalase from heat inactivation if the enzyme and its substrate are contiguous. But if they are contiguous, the question of why trehalose is not hydrolyzed in dormant ascospores remains, along with the deleterious effects of the cytoplasm on the enzyme. An alternative that will be considered in the next paper in this series is that trehalase is located outside the cell membrane but within the cell wall, as are other hydrolases in *Neurospora* (21). If this is true, the ability of trehalose to protect trehalase may be coincidental and other protective mechanisms must be sought.

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