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PURIFICATION AND PROPERTIES OF TREHALASE FROM THE THERMOPHILIC FUNGUS *HUMICOLA LANUGINOSA*

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Summary

Trehalase (α,α-Trehalose glucohydrolase, EC 3.2.1.28) was partially solubilized from the thermophilic fungus *Humicola lanuginosa* RM-B, and purified 184-fold. The purified enzyme was optimally active at 50°C in acetate buffer at pH 5.5. It was highly specific for α,α-trehalose and had an apparent K_m = 0.4 mM at 50°C. None of the other disaccharides tested either inhibited or activated the enzyme. The molecular weight of the enzyme was around 170000. Trehalase from mycelium grown at 40 and 50°C had similar properties. The purified enzyme, in contrast to that in the crude-cell free extract, was less stable. At low concentration, purified trehalase was afforded protection against heat-inactivation by "protective factor(s)" present in mycelial extracts. The "protective factor(s)" was sensitive to proteolytic digestion. It was not diffusible and was stable to boiling for at least 30 min. Bovine serum albumin and casein also protected the enzyme from heat-inactivation.

Introduction

Trehalase (α,α-Trehalose glucohydrolase, EC 3.2.1.28) from mesophilic sources has been studied previously [1—5], but there is no report on this enzyme from thermophilic organism. It was found to be one of the most active glycosidases in the common soil thermophilic fungus *Humicola lanuginosa*. In this paper the purification and properties of trehalase from this fungus are described.

Materials and Methods

Materials. Trehalose, maltose, cellobiose, lactose, melibiose, turanose, raffinose, Nitroblue Tetrazolium, phenazine methosulfate, bovine serum albumin, protamine sulfate, DEAE-cellulose (coarse), urease (jackbean), catalase (beef liver), glucose oxidase (*Aspergillus niger*), peroxidase (horseradish), pepsinogen

and trypsin were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Sephadex G-50 (coarse), Sephadex G-200 and Sepharose-4B were products of Pharmacia Fine Chemicals, Uppsala, Sweden. Rabbit muscle lactate dehydrogenase was from British Drug House, U.K. Trehalose 6-phosphate was a gift from Dr. Donald MacDonald, Orgeon State University, Oreg., U.S.A. Cow α-S-casein was a gift from Professor Paul J. Vithayathil, Indian Institute of Science, Bangalore.

Organism and growth conditions. The strain RM-B of the thermophilic fungus, *Humicola lanuginosa*, was used in the present investigation. It was isolated from horse-dung and maintained on YpSs agar-slants [6]. The culture medium contained 2% glucose, 0.4% L-asparagine, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7\ H_2O$ and 0.1 ml/l of a trace element stock solution [7]. The pH of the medium before autoclaving was 7.2 and incubation was at 50°C.

Preparation of acetone/butanol-dried mycelial powder of H. lanuginosa. 11-day-old mycelium from stationary culture or 5-day-old mycelium from shake-culture (when the specific activity of trehalase was maximal) was washed and then homogenized three times with acetone at −20°C in a Waring blendor, followed by homogenizing twice with *n*-butanol at −20°C and once with acetone at −20°C. The acetone/butanol-treated dry mycelial powder was stored at −20°C until use.

Trehalase assay. Trehalase was assayed by the method of Nelson [8] after the hydrolysis of α,α-trehalose. The reaction mixture (total volume 2 ml) contained 1.6 ml 50 mM sodium acetate buffer (pH 5.5), 0.2 ml 20 mM trehalose and 0.2 ml enzyme solution and the mixture was incubated at 50°C for 15 min.

1 unit of trehalase activity is defined as the amount of enzyme which liberates 1 nmol of glucose per min at 50°C. Specific activity is expressed as units/mg protein.

Protein estimation. Protein was estimated according to the method of Lowry et al. [9] with bovine serum albumin as standard. A micro-method of estimating protein, modified from Lowry's procedure, was also used. Up to 30 μg protein in 0.2 ml solution were mixed with 1 ml alkaline copper reagent. After 10 min, 0.1 ml Folin-Ciocalteau reagent was added and the absorbance was measured at 750 nm after 30 min.

Enzyme purification. The protocol of a typical purification procedure is described. 10 mM sodium/potassium phosphate buffer (pH 6.0) was used throughout and all operations were carried out at 0—4°C. Mycelial powder was stirred overnight with 20 vols. buffer and then centrifuged at 12000 × *g*, for 15 min. The supernatant was treated with protamine sulfate to remove nucleic acids. The enzyme solution was desalted and applied to a 45 × 1.6 cm DEAE-cellulose column equilibrated with buffer. The column was washed successively with 200 ml buffer and then with 200 ml buffer/50 mM NaCl and finally with a linear salt gradient composed by 200 ml each of 50 and 500 mM NaCl in buffer (Fig. 1). Active fractions were pooled, lyophilized and chromatographed on a 93 × 2 cm Sepharose-4B column equilibrated with buffer (Fig. 2). Active fractions were again pooled and rechromatographed on DEAE-cellulose. The enzyme was eluted with a linear salt gradient composed of 50 ml buffer and 50 ml buffer/50 mM NaCl. Active fractions were pooled and dialysed overnight against buffer.

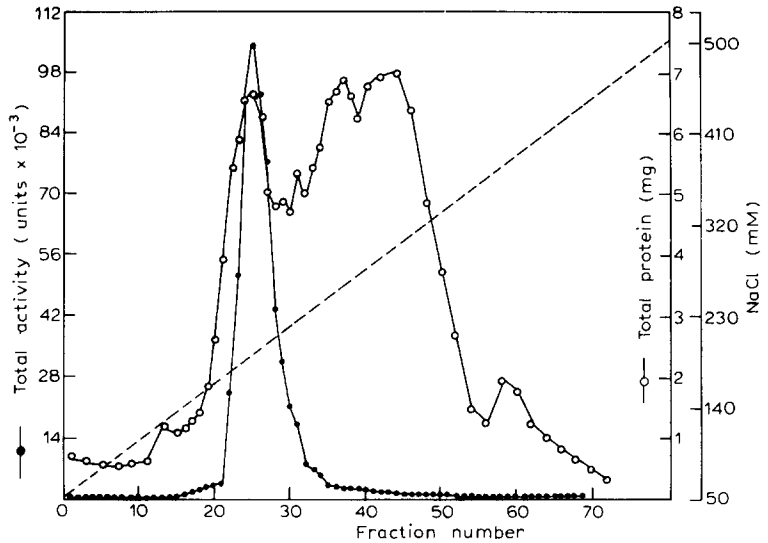

Fig. 1. Chromatography of trehalase on a 45 X 1.6 cm DEAE-cellulose column. Trehalase was eluted using a 50—500 mM linear gradient of NaCl. Fractions of 5 ml were collected at a flow rate of 60 ml/h.

Electrophoresis. Electrophoresis of the enzyme solution was carried out on 7.5% polyacrylamide gels according to the method of Davis [10].

(i) Protein staining. After electrophoresis, the gels were stained for protein with Coomassie Brilliant Blue (0.25% solution in 7% acetic acid). Gels were destained in methanol/acetic acid/water (43 : 7 : 50, v/v).

(ii) Localization of trehalase. Localization of trehalase on gels was done by the method of Eilers et al. [11].

Molecular weight determination. Molecular weight of the purified trehalase was determined by gel-filtration on a 105 X 2.5 cm Sephadex G-200 column

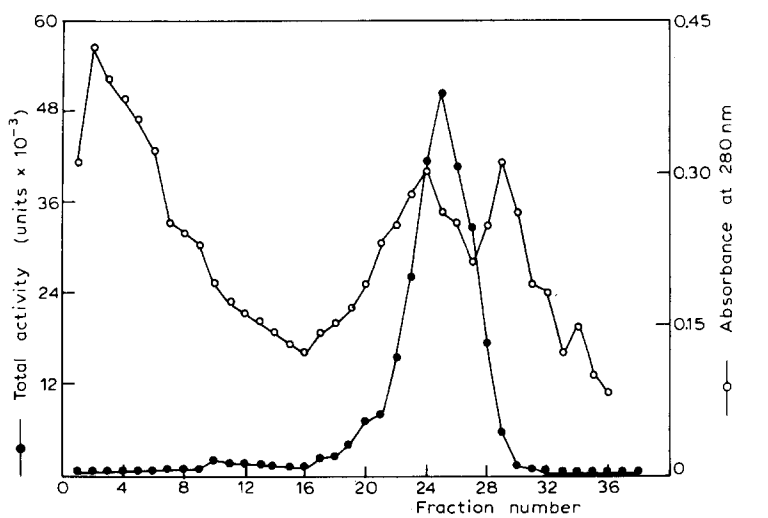

Fig. 2. Gel-filtration of trehalase on 93 X 2 cm Sepharose-4B column. After passage of 140 ml of buffer (void volume 70 ml), 2 ml fractions were collected at a flow rate of 8 ml/h.

[12]. The gel was equilibrated with 10 mM sodium/potassium phosphate buffer, pH 6.0. The following enzymes were used as markers: jackbean urease (483 000 daltons), beef liver catalase (240 000 daltons), rabbit muscle lactate dehydrogenase (151 000 daltons) and horseradish peroxidase (40 000 daltons)). The activity of each marker protein was followed by assaying for activity by methods described in the Worthington manual [13].

Heat-inactivation of trehalase. 5 ml 50 mM sodium acetate buffer (pH 5.5) containing 250—500 units trehalase were placed in a tube and immersed in a water bath. After known intervals, 0.2-ml aliquots were withdrawn into test tubes containing 1.6 ml assay buffer, kept immersed in ice. Trehalase was assayed at 50°C after adding 0.2 ml 20 mM trehalose.

Results

Extraction of trehalase

Preparation of cell-free extract from mycelium of *H. lanuginosa* presented difficulties. Usual techniques, such as mechanical homogenization, blending, grinding frozen mycelia, or sonication did not appreciably rupture cells. Extraction of mycelium with buffer after treatment with acetone and butanol butanol resulted in partial solubilization of the enzyme. Stirring this mycelial powder with buffer (10 mM sodium/potassium phosphate buffer, pH 6.0) overnight resulted in extraction of up to 50% of the total activity. Trehalase was extracted to a comparable extent from acetone/butanol-treated mycelial powder from stationary or shaker-grown cultures although approx. 3 times more protein was extracted from shaker-grown cultures.

Enzyme purification

The results of the purification are summarized in Table I. At the final stage of purification, the specific activity of trehalase was 126300 units/mg protein, representing 184-fold purification. Because of the low recovery of the enzyme, attempts to purify it further were not made.

Purity of the enzyme

Purity of the final enzyme preparation was tested by electrophoresis. 100 μg protein per gel were electrophoresed. The enzyme was not homogeneous although the major protein band coincided with the trehalase activity band.

Properties

Hydrolysis of trehalose by trehalase from *H. lanuginosa* was stoichiometric with the liberation of 2 mol glucose per mol trehalose. The reaction proceeded linearly up to at least 30 min, as shown by the liberation of increasing amounts of glucose. The amount of glucose formed was proportional to trehalase concentration up to 160 ng protein.

For studying the effect of pH on trehalase activity, 50 mM sodium acetate buffer for pH 4—5.8 and 50 mM sodium/potassium phosphate buffer for pH 5.5—8.0 were used. Trehalase showed maximal activity at pH 5.5 and was more active in acetate buffer than in phosphate buffer.

The temperature optimum for trehalose hydrolysis was determined by com-

TABLE I

SUMMARY OF PURIFICATION OF TREHALASE FROM *HUMICOLA LANUGINOSA*

Step		Total units	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Purification (-fold)
1	Crude extract	865 800	1263	685	100	1
2	Protamine sulfate precipitation	844 800	1156	731	97	1.07
3	Desalting on Sephadex G-50	755 800	583	1 296	87	1.90
4	DEAE-cellulose chromatography	340 000	22.57	15 064	39	22
5	Sepharose-4B gel filtration	194 500	2.971	65 450	22	96
6	Rechromatography on DEAE-cellulose	88 170	0.698	126 300	10	184

paring the reaction rates at 40—65°C in 50 mM sodium acetate buffer (pH 5.5). No correction was made for variation of pH with temperature. The optimum temperature for catalysis was 50°C.

The apparent K_m for trehalose at 50°C was 0.4 mM. The enzyme was specific for trehalose. Cellobiose, lactose, maltose, melibiose, raffinose, sucrose, turanose and trehalose 6-phosphate were not hydrolysed. These sugars at 1 and 5 mM concentration neither inhibited nor activated the reaction catalyzed by trehalase.

Two separate determinations of molecular weight with the same preparation of the enzyme gave values of 177800 and 167900 daltons, respectively.

There was no loss in trehalase activity when stored at 0°C for at least 20 days.

Effect of growth temperature on the heat-inactivation of trehalase

To test whether growth temperature affected the heat stability of trehalase produced in *H. lanuginosa*, a comparison was made of enzyme from 40 and 50°C-grown mycelium. Acetone/butanol-treated mycelium powder was prepared from static cultures of the fungus grown at 40°C (15 days) and at 50°C (11 days). Enzyme was purified up to the Sepharose-4B step. Purified trehalase preparation from 40°C cultures had a specific activity of 18894 and that from 50°C cultures 111500 units/mg protein. The two preparations of the enzyme were identical with respect to temperature optimum (50°C), pH optimum (5.5), migration on a Sepharose-4B column and electrophoretic mobility on polyacrylamide gel. Thermal inactivation of the two enzyme preparations was determined as a function of time at 60°C at a protein concentration of 1.7 μg/ml and was found to be similar. The two preparations responded similarly when incubated at various temperatures for 1 h as shown in Fig. 3. This showed that the cultural temperature did not affect the heat stability of the trehalase produced.

Stability of trehalase as affected by factors present in the cell

Trehalase in the buffer extracts of the acetone/butanol-treated mycelial powder was fully stable for at least 6 h at 60°C. When this extract was processed for purification, the enzyme obtained after DEAE-cellulose chromatography

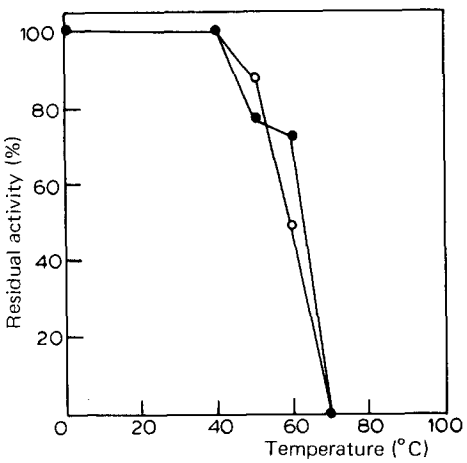

Fig. 3. Heat-inactivation of trehalase from *Humicola lanuginosa* as a function of temperature. Trehalase was incubated for 1 h at various temperatures. o———o, trehalase from mycelium grown at 50°C. ●———●, trehalase from mycelium grown at 40°C.

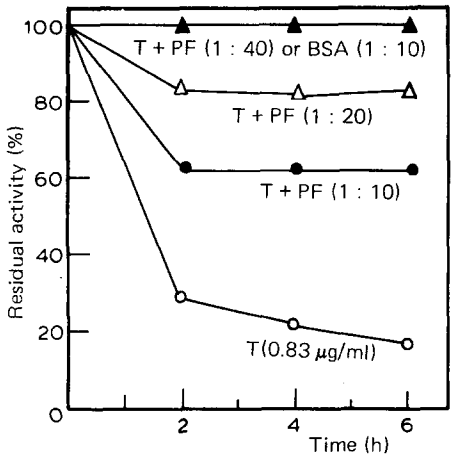

Fig. 4. Heat-inactivation of trehalase (T) in presence of "protective factor(s)" (PF) isolated from *Humicola lanuginosa* or bovine serum albumin (BSA). Purified trehalase was mixed with "protective factor(s)" or BSA at concentrations indicated.

was less stable. This suggested that the material stabilizing trehalase in the crude mycelial extracts of the thermophilic fungus was removed during this purification step.

(i) Isolation of the factor(s). Different fractions from DEAE-cellulose chromatogrpahy were lyophilized and then dialysed. The non-diffusible material was tested for the ability to induce heat stability to purified trehalase. The fraction which was unadsorbed on the DEAE-cellulose column and obtained in the buffer wash possessed the property of inducing heat stability to trehalase. It protected trehalase fully against heat-inactivation for at least 6 h at 60°C at a concentration 40 times the trehalase protein (Fig. 4). This fraction, containing "protective factor(s)", was heterogeneous having at least 10 proteins as shown by polyacrylamide gel electrophoresis.

(ii) Nature of the "protective factor(s)". (a) Effect of boiling: Aliquots of the fractioncontaining "protective factor(s)" at a concentration of 200 μg total protein in 1 ml were placed in a boiling water bath for known intervals of time. After boiling, acetate buffer (pH 5.5) and 500 units of trehalase were added to each tube to a final volume of 10 ml. 5 ml of this was used to test the effect on heat stability of trehalase at 60°C. The "protective factor(s)" was stable to boiling for at least 30 min.

(b) Effect of dialysis: The "protective factor(s)" fraction was dialysed (using a dialysis tubing with a cut-off of 12 000 daltons) against 2 l of 10 mM sodium potassium phosphate buffer (pH 6.0) for 24 h. The non-diffusible material induced heat stability to trehalase suggesting the macromolecular nature of the factor(s).

(c) Effect of proteolytic enzymes: To investigate whether the "protective factor(s)" is proteinaceous, it was treated with pepsin obtained by converting its zymogen form to active enzyme.

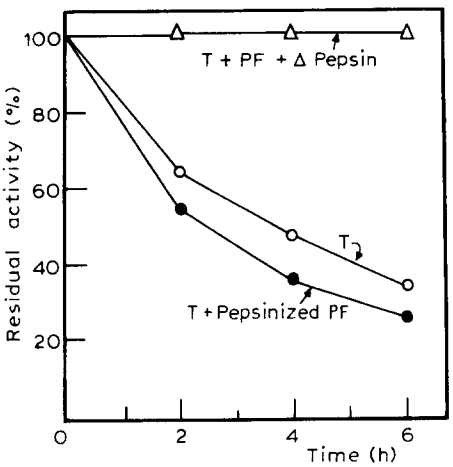

Fig. 5. Heat-inactivation of trehalase (T) in the presence of pepsin-treated "protective factor(s)" (PF). Details as in the text.

The "protective factor(s)" fraction containing 200 μg total protein was mixed with 0.5 ml 0.3 M HCl and incubated at 37°C (conversion of pepsinogen to pepsin is autocatalytic at acid pH). Pepsinization of the factor was started by the addition of 3 units (0.1 ml) of pepsinogen. After 1 h, 0.5 ml 0.15 M NaOH was added to neutralize the acid and pepsin was inactivated by boiling for 30 min. Trehalase (500 units) was added to the above tube and diluted to 10 ml with acetate buffer. 5 ml of this solution was used in testing the ability of pepsin-treated factor in inducing stability to trehalase at 60°C. Pepsin-treatment of the fraction containing "protective factor(s)" destroyed its property of protecting trehalase against inactivation at 60°C (Fig. 5). In contrast, trehalase treated with "protective factor(s)" and "protective factor(s)" treated with heat-inactivated pepsin remained stable for at least 6 h at 60°C. Similar results were obtained, when the "protective factor(s)" was treated with trypsin. These results suggested the proteinaceous nature of the "protective factor(s)".

Effect of extraneous proteins on the heat-inactivation of trehalase

Heat-inactivation of trehalase in the presence of bovine serum albumin was studied. Purified trehalase was mixed with bovine serum albumin in acetate buffer and incubated at 60°C for known intervals of time. Bovine serum albumin at 10 times the trehalase concentration afforded protection against heat-inactivation to *Humicola* enzyme (Fig. 4).

Purified cow α-*S*-casein also protected trehalase against heat-inactivation.

Discussion

In general, purification of trehalase from almost all sources has been difficult, the recoveries being low. Trehalase has been partially purified from various microbial sources using the conventional methods such as $(NH_4)_2SO_4$ or acetone fractionation, chromatography on DEAE-cellulose and hydroxyapatite [1,2,14]. Significant losses occurred when $(NH_4)_2SO_4$ or acetone fractionation

were used as purification steps in the present study. Recently, Kelly and Catley [5] have reported a purification of *Saccharomyces cerevisiae* trehalase by affinity chromatography on concanavalin A-Sepharose. However, they did not give any data on the state of purity of the enzyme. The only reports of purification of trehalase to homogeneity are those of Lefebvre and Huber [15], Huber and Lefebvre [16], Talbot et al. [17] from insects and Nakano et al. [18] from rat intestinal mucosa.

The pH optimum of trehalase from *H. lanuginosa* is in general agreement with that reported from other sources where it has ranged from pH 5—6 and is close to that of trehalase from *Neurospora* [1], baker's yeast [2], *Dictyostelium discoideum* [14] and *Drosophila melanogaster* [19].

The optimum temperature for trehalose hydrolysis by trehalase from *H. lanuginosa* is 50°C which is similar to that reported from other mesophilic sources such as yeast [2], *D. discoideum* [14], insects [16,17].

Trehalase from *H. lanuginosa* like this enzyme from other sources, is highly specific for trehalose, with K_m = 0.4 mM at 50°C. Reported K_m values for trehalose have ranged from 0.2—20 mM [20]. Courtois et al. [21] and Guilloux et al. [22] have made extensive studies on the specificity of trehalase purified from insects. These workers used various substrate analogues and concluded that free hydroxyl groups at C_3 and C_6 to be essential for enzymatic activity. Many disaccharides and substrate analogues act as inhibitors for trehalase purified from other sources. Trehalose 6-phosphate acted as a competitive inhibitor for the intracellular trehalase of yeast and glucose acted as a non-competitive inhibitor [23]. Trehalosamine acted as an inhibitor for trehalase from *Streptomyces hygroscopicus* [24] and *Melanopus differentialis* [25]. Mannitol, present as a reserve material in *Aspergillus oryzae*, acted as a competitive inhibitor for trehalase [3]. Mannitol occurs in the mycelium of *H. lanuginosa*, but it did not act as an inhibitor for *H. lanuginosa* trehalase.

The average molecular weight of trehalase from *H. lanuginosa* as determined by gel-filtration on Sephadex G-200 was approx. 170000. Other workers have reported values of 90000—400000 daltons [4,15—18,21,22,26].

It has been reported that thermal stability of enzymes may be determined by the temperature of growth. For example, purified amylase from *Bacillus stearothermophilus* [27,28] and *B. coagulans* [29], neutral protease and pyrophosphatase from *B. stearothermophilus* [30,31] were more stable when the organism was cultured at higher temperature. Haberstich and Zuber [32] found several enzymes to be more stable in the extracts of *B. stearothermophilus* and *B. caldotenax* grown at 50°C than at 37°C. In *H. lanuginosa*, temperature of growth did not affect the heat stability of trehalase. This finding is in accord with the observation made for other enzymes [33—36].

In mesophilic fungi, trehalase from *Neurospora crassa* [26] is the most stable. The thermophilic fungus, *H. lanuginosa*, did not produce trehalase of extra thermostability. The stability of the *H. lanuginosa* enzyme was markedly enhanced by the presence of proteinaceous factor(s) obtained from the cell or, by extraneous proteins such as bovine serum albumin and casein. This observation is significant as it shows that mechanisms other than the innate resistance of proteins exist, enabling the growth and survival of thermophiles at high temperatures.

Acknowledgement

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