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Purification and properties of an extracellular conidial trehalase from *Humicola grisea* var. *thermoidea*

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An extracellular trehalase (α,α-trehalose glucohydrolase, EC 3.2.1.28) was purified from conidia of *Humicola grisea* var. *thermoidea*. The purified enzyme is a glycoprotein and migrates as a single polypeptide band during polyacrylamide gel electrophoresis under non-denaturing conditions. The apparent molecular weight of the enzyme was estimated as 580 000 by gel filtration chromatography. The enzyme is separable into three polypeptide bands of 105 000, 98 000 and 84 000 daltons on SDS-PAGE. It is specific for trehalose and its activity is not inhibited by other dissaccharides. It has a K_m of 2.3 mM, an optimum pH of 5.6 in sodium acetate buffer and a temperature optimum of 60 °C. The enzyme is activated by Ca^{2+}, Co^{2+} and Mn^{2+} and inhibited by inorganic phosphate, AMP, ADP or ATP. The inhibitory effect of phosphate, AMP and ADP, but not that of ATP, was abolished in the presence of Ca^{2+}.

Introduction

The dissaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is found in a variety of organisms including bacteria, yeasts, filamentous fungi, plants and insects [1] where it serves as endogenous carbon reserve for a number of physiological functions [2–7]. Many cells accumulate trehalose in preparation for dormancy [8,9] and in this case in addition to its role as a carbon reserve, trehalose also appears to maintain the integrity of biological membranes in the dry state [8,10]. Thus, it has been suggested that trehalose by interacting with the polar head groups of membrane phospholipids [8,11,12] maintains the membrane in its hydrated configuration in the absence of water.

Trehalose is hydrolysed to glucose by the enzyme trehalase (EC 3.2.1.28). The biochemical properties of this enzyme, which has been isolated from different sources including bacteria, filamentous fungi and yeasts, plants, insects and mammals, are highly variable [1]. In view of the important biological role of trehalose, the use of the enzyme trehalase may be of technological and academic interest for the quantification of trehalose in cells or organic materials.

Among the filamentous fungi the genus *Humicola* has been described as a good source of glycosidase activities [13–15] and the intracellular trehalase from *Humicola lanuginosa*, highly specific for trehalose has been isolated and partially characterized [14].

Preliminary observations from our laboratory have demonstrated that the thermophilic fungus *Humicola grisea* var. *thermoidea* is a rich source of extracellular trehalase activity, present on the surface of conidia. In the present report, we characterize this enzyme biochemically.

Materials and Methods

Organism and growth conditions

The *Humicola* strain utilized in this study was isolated from Brazilian soil and identified as *H. grisea* var. *thermoidea* on the basis of morphology and cultural characteristics, according to Cooney and Emerson [16]. The fungus was grown at 40 °C on solid, 4% oatmeal (Quaker) medium. Conidia, harvested from 10-day-old cultures were used as the source of trehalase activity.

Extraction and purification of conidial trehalase

The mould was grown as described above, in 250 ml Erlenmeyer flasks containing 50 ml of solid culture medium. 5 ml of chilled distilled water was added to each flask, and the surface of the culture was gently

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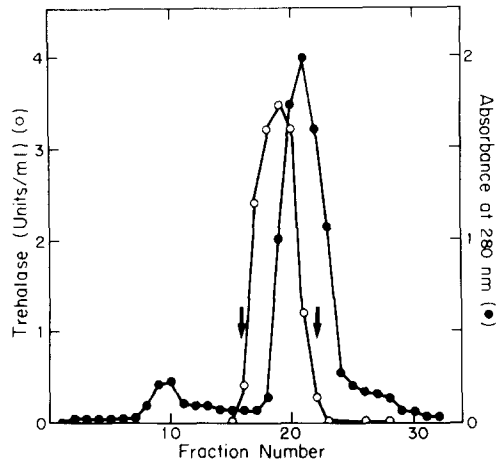

Fig. 1. Gel filtration of trehalase on Fractogel 65 (S) column. The kaolin eluate was concentrated by lyophilization, dissolved in 50 mM sodium phosphate buffer (pH 6.0), 1 mM EDTA and 1 mM sodium azide. The sample (5.0 ml) was added to a Fractogel (40×3 cm) column and eluted with phosphate buffer. Fractions (5.0 ml) were collected at a flow rate of 15 ml/h.

scraped with a spatula to suspend conidia formed on the mycelium surface. After 30 min on ice with occasional agitation, the conidial suspension was passed through two layers of gauze and centrifuged for 5 min at $10000 \times g$. The supernatant containing 90% of the total trehalase activity was used as a source of the crude enzyme. Routinely, 600 mg (dry weight) of conidia was used for each enzyme preparation. The crude enzyme solution was treated with kaolin (1 g/100 ml) for 10 min, in an ice bath, to remove pigments. The suspension was then centrifuged and the supernatant fraction was lyophilized, dissolved in 5 ml of 50 mM sodium phosphate buffer (pH 6.0) plus 1 mM sodium azide and 1 mM EDTA (phosphate buffer) and added to a Fractogel 65 (S) (40 × 3 cm) column equilibrated and eluted with phosphate buffer (Fig. 1). Fractions from the column containing trehalase activity were pooled and added to a DEAE-cellulose column (21 × 1.5 cm) equilibrated with phosphate buffer and eluted with a linear phosphate buffer gradient (50–500 mM) at pH 6.0 (Fig. 2). After this step the enzyme preparation appeared homogeneous (see Results).

Enzyme assay

Routinely trehalase activity was assayed with trehalose as a substrate by determining the amount of reducing sugar released as follows: 1 ml of 50 mM sodium acetate (pH 5.6) containing 5 mg trehalose and 1 ml of conveniently diluted enzyme were incubated for 10–20 min at 60 °C and the glucose liberated was measured as a reducing sugar [17]. When substrate specificity or inhibition by other carbohydrates was investigated, glucose was determined by the glucose oxidase assay [18].

Apparent K_m values were determined by linear regression analysis of double-reciprocal plots, obtained from four independently purified enzyme batches. Each plot consisted of at least eight data points obtained over a range of substrate concentrations from 1.0 to 20 mM. Each point was the mean value of three experiments. A trehalase unit was defined as the amount of enzyme which released 1 μmol of glucose per min at 60 °C.

Polyacrylamide gel electrophoresis

Electrophoresis of the purified enzyme was carried out in 7.5% polyacrylamide rod gels according to Davis [19]. Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Weber and Osborn [20] using 5% acrylamide. Proteins were stained with Coomassie blue. The following molecular weight standards were used: myosin (200000); phosphorylase (97400); and bovine serum albumin (66000).

Sepharose-4B column chromatography

The molecular weight of the purified enzyme was estimated by Sepharose-4B gel filtration. A sample of enzyme (0.5 ml) was loaded onto a Sepharose-4B column (72 × 1.0 cm) equilibrated with 50 mM sodium phospahte buffer (pH 6.0) containing 100 mM NaCl. The following proteins were used as standards: thyroglobulin (670000); urease (480000); and catalase (230000). The column was eluted at 5 °C at a flow rate of 10 ml per h. The eluate was collected in 2.0 ml fractions.

Determination of neutral carbohydrate and protein

Total neutral carbohydrate was estimated by the phenolsulphuric acid method of Dubois et al. [21] using glucose as a standard. Protein was determined by the

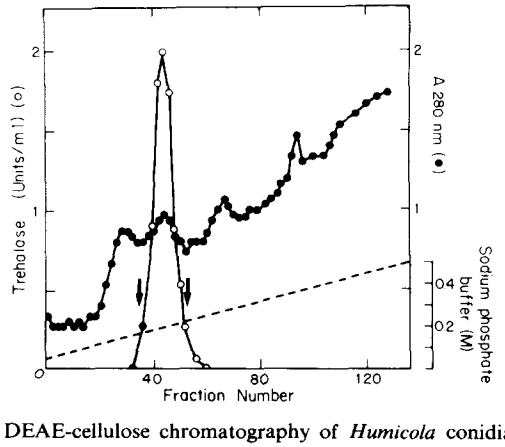

Fig. 2. DEAE-cellulose chromatography of *Humicola* conidia trehalase. Trehalase activity eluted from Fractogel 65 (S) was applied to a DEAE-cellulose column (21 × 1.5 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.0), 1 mM EDTA and 1 mM sodium azide, and eluted with a sodium phosphate buffer gradient (50–500 mM) at flow rate of 30 ml/h.

method of Lowry et al. [22] using bovine serum albumin as a standard.

Reagents

Trehalose, AMP, ADP, ATP, thyroglobulin, urease, catalase, bovine serum albumin, myosin, phosphorylase and DEAE-cellulose were purchased from the Sigma Chemicals St. Louis, MO, U.S.A. Sepharose-4B was obtained from Pharmacia (Uppsala, Sweden). Glucose oxidase reagents were obtained as a commercial kit prepared by Labtest (Belo Horizonte, Brasil). All other chemicals were of the highest purity available.

Results

Purification and biochemical characterization of conidial trehalase activity

Table I summarizes the data of a typical purification of conidial trehalase from *H. grisea* var. *thermoidea.* After DEAE-cellulose chromatography, the specific activity of trehalase was 73.0 units per mg of protein and a purification of 8.1-fold was achieved. Non-denaturing, polyacrylamide gel electrophoresis of the purified enzyme showed a single protein band stained with Coomassie blue (Fig. 3A). However, under denaturing conditions (SDS-PAGE) the purified enzyme produced three polypeptides (Fig. 3B) which migrated to show apparent molecular weights of 105000, 98000 and 84000, respectively (Fig. 4B). Gel filtration on Sepharose-4B indicated that the native purified enzyme had an apparent molecular weight of approx. 580000 (Fig. 4A).

The carbohydrate content of the purified enzyme was estimated as 56%, using glucose as a standard.

Catalytic properties

The effect of several cations on trehalase activity was investigated (Table II). Activation of trehalase was observed in the presence of calcium chloride (4.7-fold), manganese chloride (4.1-fold) and cobalt chloride (2.8-fold).

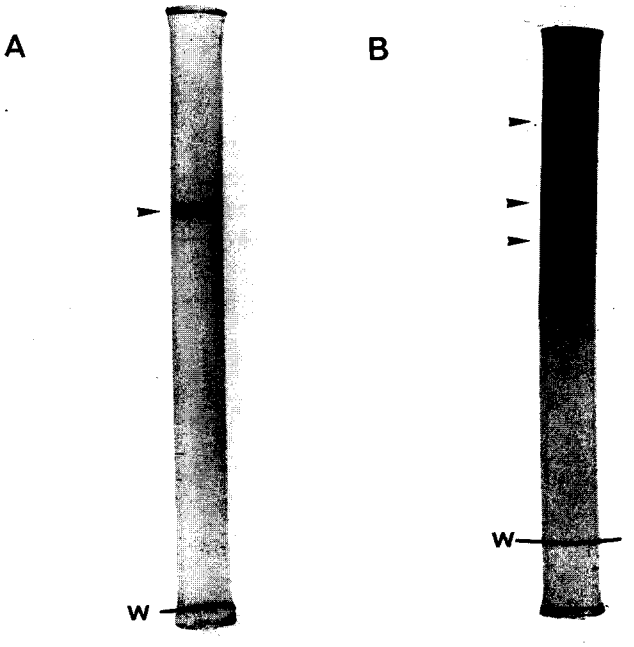

Fig. 3. Gel electrophoresis of purified trehalase. A piece of wire (W) indicates the position of the marker dye front. The gels were stained with Coomassie blue. Enzyme bands are indicated by the arrows. (A) Polyacrylamide gel electrophoresis at pH 8.9, 30 μg protein was applied. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis, 100 μg protein was applied.

The effect of pH on trehalase activity was investigated using 50 mM sodium acetate (pH 3.0–6.0) and 50 mM Tris-HCl (pH 6.0–10.0) buffers. Trehalase showed maximal activity at pH 5.6 with or without calcium chloride 5 mM (Fig. 5A). When acetate buffer at pH 5.6 was replaced by sodium phosphate buffer, the enzyme activity decreased 50%. The optimum temperature for trehalase activity was determined by comparing the reaction rates at different temperatures from 25 to 80 °C in sodium acetate buffer at pH 5.6. The optimum temperature for trehalose hydrolysis was 60 °C, both in the presence and absence of 5 mM calcium (Fig. 5B).

TABLE I

Purification of conidia trehalase from H. grisea var. thermoidea

Step [a]	Total volume (ml)	Total protein (mg)	Total units	Specific activity (U/mg protein)	Enzyme yield (%)	Purification (-fold)
Crude enzyme (conidial wash supernatant)	175	22.2	202.9	9.1	100	1.0
Kaolin eluate	172	11.0	122.6	11.1	60	1.2
Fractogel	33	6.3	51.6	8.2	25	0.9
DEAE-cellulose	30	0.4	29.6	73.9	14.6	8.1

[a] 600 mg (dry weight) of *H. grisea* conidia were used as a source of crude enzyme. Other details are given in Material and Methods. Enzyme units are defined as the amount of enzyme which releases 1 μmol glucose per min, at 60 °C.

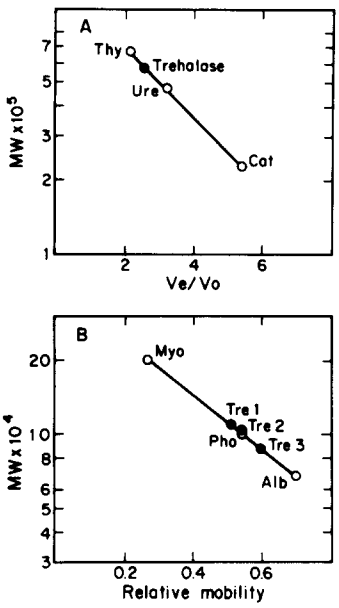

Fig. 4. Estimation of molecular weight of the purified trehalase. (A) Standards for molecular weight estimation in Sepharose-4B: thyroglobulin (Thy); urease (Ure); and catalase (Cat). (B) Standards for molecular weight estimation by sodium dodecyl sulphate electrophoresis: myosin (Myo); phosphorylase (Pho); bovine serum albumin (Alb); and trehalase (Tre). See Materials and Methods for details.

TABLE II

The effect of various cations on trehalase activity

Cation	Trehalase activity (units/ml)	Activation (-fold)
Control	1.2	-
$CaCl_2$ (5 mM)	5.6	4.7
$MgCl_2$ (10 mM)	1.3	1.1
$MnCl_2$ (7.5 mM)	4.9	4.1
$CoCl_2$ (1 mM)	2.8	2.3
NH_4Cl (1 mM)	1.0	0.8
$CuCl_2$ (1 mM)	0.8	0.7
$ZnCl_2$ (1 mM)	0.8	0.7
Al_2Cl_3 (1 mM)	1.5	1.2
$BaCl_2$ (1 mM)	1.3	1.1

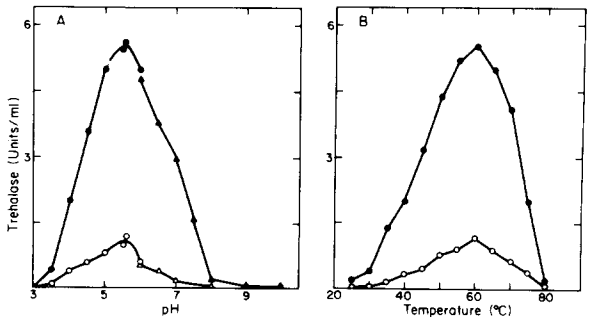

Fig. 5. The effect of pH on trehalase activity. At pH 3.0–6.0 (sodium acetate, with (●) and without (○) calcium); and pH 6.0–10.0 (Tris-HCl, with (▲) and without (△) calcium). (B) The effect of temperature on trehalase activity, with (●) and without (○) calcium.

TABLE III

Inhibition of Humicola purified trehalase by nucleoside phosphate

Nucleoside phosphate (10 mM)	$CaCl_2$ (mM)	Trehalase activity (units/ml)	Inhibition (%)
None	0	1.2	-
AMP	0	1.0	17
ADP	0	0.5	58
ATP	0	0.6	50
None	5.0	5.6	-
AMP	5.0	5.2	7
ADP	5.0	2.6	54
ATP	5.0	0.8	91

Adenosine triphosphate is a known inhibitor of trehalase activity in *Saccharomyces cerevisiae* [23]. However, the trehalase purified from *Humicola* was markedly inhibited by inorganic phosphate. To ascertain whether nucleoside phosphates might similarly affect the activity of the *Humicola* trehalase, the effect of AMP, ADP and ATP on the enzyme activity was tested. At a concentration of 10 mM, AMP, ADP and ATP inhibited trehalase activity by about 17, 58 and 50%, respectively (Table III). The inhibitory effects of ADP and ATP were also observed when the enzyme was activated 4.7-fold in the presence of calcium. In this case, ATP completely abolished the calcium activating effect while ADP, which inhibited about 50% of the enzyme activity, did not interfere with the calcium activating effect (Table III).

The purified *Humicola* trehalase was highly specific for trehalose as a substrate and did not hydrolase cellobiose, lactose, maltose, raffinose or sucrose. Trehalose hydrolysis was not affected by the presence of D-mannitol, fructose or maltose at 10 mM concentration.

Kinetic studies

Comparative studies were performed to determine the K_m and V_{max} of *Humicola* trehalase in the presence or absence of calcium or manganese. Table IV shows that the apparent K_m values for the enzyme were essentially the same. However, calcium or manganese

TABLE IV

Kinetic constants of trehalase purified from Humicola conidia

Treatment	K_m (mM)	V_{max} (units/mg protein)
Control	2.3 ± 0.2	176 ± 19
$CaCl_2$ (5 mM)	2.5 ± 0.1	521 ± 24
$MnCl_2$ (7.5 mM)	2.4 ± 0.2	481 ± 29
ATP (5 mM)	5.3 ± 0.9	118 ± 21
ATP (10 mM)	8.7 ± 1.3	108 ± 12

increased V_{max} 3.0- and 2.7-fold, respectively. The inhibitory effect of ATP suggested a mixture of competitive and non-competitive mechanisms since K_m was increased and V_{max} values lowered (Table IV).

Discussion

The purification procedure described here was based on conventional techniques, following solubilization of trehalase activity from *Humicola* conidia by simply washing with water. Thus, *Humicola* conidia may be considered as an excellent source of trehalase activity because the enzyme is easily removed from the cells and is relatively free of protein contaminants compared to other intracellular enzyme preparations.

The purification of trehalase from almost any source is reportedly difficult due to low enzyme recoveries [14]. Furthermore, little is known about trehalase from thermophilic soil fungi [14], particularly trehalase activity present in conidia. The specific activity of the purified *Humicola* trehalase preparation when activated by calcium was 2.8-times higher than that reported for partially purified *H. Lanuginosa* trehalase [14], *Humicola* conidia trehalase was very specific for trehalose and remained stable for more than 12 months at −20 °C. The purification procedure yielded a homogeneous enzyme preparation with an apparent molecular weight of 580000 as ascertained by non-denaturing, polyacrylamide gel electrophoresis and by gel filtration in Sepharose-4B. However, using SDS-PAGE, the purified enzyme was resolved into three polypeptides with molecular weights of 105000, 98000 and 84000. These results suggest that the native enzyme may be a hexameric protein composed of three classes of polypeptide of different molecular weights. Trehalase from *Neurospora crassa* [24], *S. cerevisiae* [25], *Phycomyces blakesleeanus* [26] and *H. lanuginosa* [14] exhibit molecular weights of 400000, 320000, 210000 and 170000, respectively. The apparent molecular weight of the *H. grisea* trehalase (580000) should be taken as an approximation since the enzyme binds irreversibly to Con A-Sepharose (data not shown) and had a high carbohydrate content (56%).

The optimum pH for *Humicola* conidia trehalase was 5.6, similar to those reported for other fungal trehalases which lie between pH 5–6 [14,24,27,28]. The optimum temperature for *Humicola* conidia trehalase activity was 60 °C, about 10 °C higher than those of trehalases from mesophylic fungi reportedly having the highest temperature optima, such as trehalases from *S. cerevisiae* [5], *Dictyostelium discoideum* [29] or even the thermophylic organism *H. lanuginosa* [14].

Humicola conidia trehalase was activated by calcium, manganese and cobalt. The calcium effect did not appear to be related to enzyme protection against thermal inactivation because calcium activation was also observed at low temperatures (Fig. 5B). Activation effects of divalent cations also have been reported for one of the trehalases purified from yeast [28]. The increase in trehalase activity seen during incubation with calcium, manganese or cobalt (Table II), may be related to changes in the state of aggregation of the enzyme. Aggregates of trehalase have been described for the enzyme in *N. crassa* [24]. Other examples are reported where high salt concentrations either stabilize [30,31] or labilize [32] enzymes, and in which changes in protein stability were correlated with the formation [30] or dissociation of aggregates [31,33]. Aggregation of *Humicola* conidia trehalase is likely because the concentration of the enzyme by lyophilization results in a significant increase in specific activity (data not shown). Furthermore, a highly diluted enzyme solution rapidly loses its activity in the absence of calcium (data not shown).

ATP is reported to be an inhibitor of the trehalase activity of *S. cerevisiae* [23] and *P. blakesleeanus* [26]. Trehalase from *H. grisea* conidia was similarly inhibited by ATP and to a lesser extent by ADP and AMP. The inhibitory mechanism of ATP on *H. grisea* trehalase probably differs from that of ADP, since ATP inhibition, but not that of ADP, also blocked activation of the enzyme by calcium. Thus, it seems unlikely that ATP inhibited *Humicola* trehalase by simply complexing the divalent ions necessary for enzyme activity. Moreover, the inhibition of the *Humicola* trehalase activity caused by ATP was of a mixed type, affecting both K_m and V_{max}.

The K_m values of non-activated and calcium-activated *Humicola* trehalase (2.3 and 2.5 mM, respectively) are comparable to those of other trehalases for which K_m values range from 0.2 to 20 mM [1], but were higher than that of the *H. lanuginosa* enzyme which is 0.4 mM [14].

Thevelein [7] has classified the different trehalases into nonregulated or 'acid trehalases', the activity of which is not dependent on protein phosphorylation, and neutral or 'regulatory trehalases' which regulate trehalose mobilisation and are controlled by a phosphorylation/dephosphorylation mechanism. In view of its properties, the trehalase from *H. grisea* conidia can be classified as of the non-regulatory enzyme type. Indeed, all attempts to activate the *Humicola* enzyme by cyclic AMP-protein kinase mediated phosphorylation gave negative results (unpublished observation), a fact not at all surprising considering the localisation of the enzyme at the surface of the cells. The results may be of relevance to knowledge of the properties of trehalases located outside the cell permeability barrier.

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