The Journal of Microbiology (2011) Vol. 49, No. 5, pp. 809-815
DOI 10.1007/s12275-011-0532-4

Biochemical Properties of an Extracellular Trehalase from *Malbranchea pulchella* var. Sulfurea

Marita Gimenez Pereira, Luis Henrique Souza Guimarães, Rosa Prazeres Melo Furriel, Maria de Lourdes Teixeira de Moraes Polizeli, Hector Francisco Terenzi, and João Atílio Jorge*

Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes, 3900, CEP 14040-901- Ribeirão Preto, SP, Brasil

(Received December 21, 2010 / Accepted April 27, 2011)

The thermophilic fungus *Malbranchea pulchella* var. sulfurea produced good amounts of extracellular trehalase activity when grown for long periods on starch, maltose or glucose as the main carbon source. Studies with young cultures suggested that the main role of the extracellular acid trehalase is utilizing trehalose as a carbon source. The specific activity of the purified enzyme in the presence of manganese (680 U/mg protein) was comparable to that of other thermophilic fungi enzymes, but many times higher than the values reported for trehalases from other microbial sources. The apparent molecular mass of the native enzyme was estimated to be 104 kDa by gel filtration and 52 kDa by SDS-PAGE, suggesting that the enzyme was composed by two subunits. The carbohydrate content of the purified enzyme was estimated to be 19% and the pI was 3.5. The optimum pH and temperature were 5.0-5.5 and 55°C, respectively. The purified enzyme was stimulated by manganese and inhibited by calcium ions, and insensitive to ATP and ADP, and 1 mM silver ions. The apparent K_M values for trehalose hydrolysis by the purified enzyme in the absence and presence of manganese chloride were 2.70±0.29 and 2.58±0.13 mM, respectively. Manganese ions affected only the apparent V_{max}, increasing the catalytic efficiency value by 9.2-fold. The results reported herein indicate that *Malbranchea pulchella* produces a trehalase with mixed biochemical properties, different from the conventional acid and neutral enzymes and also from trehalases from other thermophilic fungi.

***Keywords*:** trehalase, extracellular trehalase, acid trehalase, neutral trehalase, *Malbranchea pulchella*

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), a non-reducing disaccharide, is hydrolyzed by the enzyme trehalase (EC 3.2.1.28). Trehalases exist in a wide variety of organisms, including bacteria, yeast, filamentous fungi, plants, insects and mammals (Elbein, 1974). For a long time, trehalose in fungi was considered as a storage carbohydrate, accumulating under conditions of imminent carbon deficiency and being mobilized in response to carbon starvation (Thevelein, 1984; Bonini *et al*., 2004; Parrou *et al*., 2005). However, several *in vivo* experiments also showed that trehalose levels closely correlate with stress resistance. Further, *in vitro* experiments also disclosed the potential of trehalose as a stabilizing agent of cell membranes and proteins (Singer and Lindquist, 1988; Simola *et al*., 2000).

The catabolism of fungal trehalose is carried out mainly by trehalase, although an alternative pathway involving trehalose phosphorylase has been found in *Pichia fermentans* (Schick *et al*., 1995) and some other species (Parrou *et al*., 2005). Traditionally, fungal trehalases have been classified as acid (or non regulatory) and neutral (or regulatory), according to the optimum pH and some regulatory properties, and coexist in a majority of fungi studied up to date (Thevelein, 1984, 1988; Jorge *et al*., 1997). Filamentous fungi neutral trehalases (unglycosylated, cytosolic proteins) show similarities with yeast neutral trehalases, being activated by cAMP-dependent phosphorylation, Ca^{2+} and Mn^{2+}, and inhibited by ATP (Panek, 1969; Thevelein, 1984, 1988; Jorge *et al*., 1997; Bonini *et al*., 2004). In contrast, acid trehalases (extracellular or vacuolar glycoproteins) are generally present at the surface of spores, mycelium or vacuoles, or, less frequently, are free in the external medium, and their synthesis is repressed by glucose (Jorge *et al*., 1997). Moreover, most filamentous fungi acid trehalases exhibit maximum activity at high temperatures, elevated thermostability, are not regulated by reversible phosphorylation neither activated by Ca^{2+} and Mn^{2+}, and are also not inhibited by ATP.

Recently, some studies on thermophilic fungi strongly suggested the existence of a new class of trehalases, exhibiting mixed properties from the acid and neutral ones (Kadowaki *et al*., 1996; Lucio-Eterovic *et al.*, 2005). In effect, thermophilic fungi trehalases are activated by Ca^{2+} and Mn^{2+} and inhibited by ATP, but not regulated by reversible phosphorylation. Further, they exhibit maximum activity at a high temperature and acidic pH, but are located at the cytosol or linked to the cell wall in the periplasmic space, or else freely secreted into the medium. In addition, data obtained with *Candida albicans* revealed two trehalases with mixed properties, classified as acid and neutral considering their cell localization and optimum pH (Sánchez-Fresneda *et al*., 2009). In fact, this data set suggests that trehalases may constitute a more heterogeneous group of glycosidases than expected in the past.

The present study describes the biochemical properties of an acid extracellular trehalase from *Malbranchea pulchella*

* For correspondence. E-mail: joajorge@usp.br; Tel.: +55-16-3602-4679; Fax: +55-16-3602-4886

var. sulfurea, produced and secreted into the culture medium in the presence of starch as the main carbon source. Although sharing properties of acid and neutral trehalases, the extracellular *Malbranchea* enzyme is inhibited by Ca^{2+} and insensitive to ATP, in contrast to other thermophilic fungi trehalases (Cardello *et al*., 1994; Kadowaki *et al*., 1996; Almeida *et al*., 1999), and, amongst other interesting properties, is also insensitive to Ag^{+}. The enzyme seems to be controlled by catabolic regulatory circuits and to be involved in the assimilation of exogenous trehalose as a source of carbon.

Materials and Methods

Microorganism and culture conditions

A *M. pulchella* var. sulfurea strain (ATCC 42653) was maintained at 40°C in slants of 4% baby food oat meal and 1.8% agar. Conidia from 10-day old cultures were inoculated into a liquid medium containing 0.4% yeast extract, 0.1% monobasic potassium phosphate, 0.05% magnesium sulfate, and 1.0% carbon source. These compounds were dissolved in 25% tap water and 75% distilled water, and the pH was adjusted to 6.0. Studies concerning the induction of the enzyme were conducted using mycelia grown in a culture medium containing glucose as the main carbon source. After a 48 h growth, the mycelia were harvested, washed with sterile distilled water and transferred to 25 ml fresh medium supplemented with 1% glucose, 1% starch or 1% trehalose. For purification procedures, *M. pulchella* was cultivated in Erlenmeyer flasks (250 ml) containing 50 ml of medium, incubated at 40°C for 96 h in a reciprocating shaker (140 rpm).

Purification of the extracellular trehalase

The mold was grown as described above and the culture filtrate (1 L) was obtained from 30 flasks after filtration on Whatman paper #1. The crude filtrate was applied to a DEAE-cellulose column (15×2.5 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0. The column was washed with 300 ml of equilibrating buffer and the proteins were eluted at a flow rate of 60 ml/h with a linear gradient of NaCl (0-500 mM) in the same buffer. A peak of trehalase activity was eluted with 220 mM NaCl. The active fractions were pooled and solid $(NH_4)_2SO_4$ was added to achieve a 1.5 M concentration. The sample containing salt was applied to a Phenyl-Sepharose CL-4B column (5.0×2.5 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0, containing 1.5 M $(NH_4)_2SO_4$. The column was washed with 150 ml of equilibrating buffer and then eluted with an inverse linear gradient of $(NH_4)_2SO_4$ (1.5-0 M). A single trehalase peak was eluted from the hydrophobic resin at a salt concentration of about 0.40-0.45 M. The active fractions were pooled, dialyzed against a 50 mM sodium acetate buffer, pH 5.0, and applied to a DEAE-cellulose column (10.0×1.5 cm) equilibrated with the same buffer. The column was washed with 140 ml of equilibrating buffer and eluted with a linear gradient of NaCl (0-0.5 M) in acetate buffer. A peak of treahalase activity was eluted when the NaCl concentration reached about 125 mM. Fractions with high activity were pooled, dialyzed and concentrated by dried freezing. After the second DEAE-column, the extracellular preparation appeared homogeneous to PAGE criteria.

Enzymatic assay

Trehalase activity was routinely assayed in 50 mM sodium acetate buffer, pH 5.0, containing 20 mM trehalose. The reaction was performed at 55°C, samples were withdrawn after convenient time intervals and the glucose released was estimated using either the DNS (Miller, 1959) or glucose oxidase (Bergmeyer and Bernt, 1974) methods. One enzyme unit (U) was defined as the amount of enzyme producing 1 μmol glucose per min. The specific activity was expressed as units per mg protein (U/mg).

Determination of kinetic parameters

The trehalase activity of the purified enzyme was determined at 55°C, in 50 mM sodium acetate buffer (pH 5.0) containing the substrate in a concentration range varying from 1 to 30 mM, in the absence and presence of $MnCl_2$ (2 and 10 mM). Kinetic parameters (V_{max} and K_M) for trehalose hydrolysis were calculated using the software SigrafW, which fits the experimental data to the Hill equation using non-linear regression (Leone *et al*., 1992). Lineweaver-Burk data fitting and statistical analyses were carried out using the OriginPro 8 SRO software package (OriginLab Corp., USA).

Electrophoresis

Electrophoresis under non-denaturing conditions was carried out according to Davis (1964) using 6% acrylamide. After PAGE, the gels were stained for protein with Coomassie Brilliant blue. When stained for activity, the gels were pre-washed for 20 min in the reaction buffer (50 mM sodium acetate, pH 5.0) containing 25% (v/v) isopropyl alcohol. Two additional washings (20 min each) were carried out in the same buffer without the alcohol. The trehalase activity was visualized after incubation of the gels for 15-20 min at 40°C in 10 ml of the reaction buffer containing 10 mg trehalose, 1 mM $MnCl_2$, 30 U glucose oxidase (Merck™, Germany), 4 mg nitroblue tetrazolium and 2 mg phenazine methosulfate. SDS-PAGE was carried out in 7% acrylamide gels, according to Laemmli (1970). The molecular mass marker was obtained from Sigma Chemical Co. (USA). Isoelectric focusing of the purified enzyme (20 μg) was carried out as described by O'Farrel *et al*. (1977) in rod gels (6% acrylamide, 0.6×13.0 cm) using Pharmalyte (pH 2.5-5.0) at a concentration of 5% (v/v). After focusing at 500 V for 6 h, the pH gradient was measured by cutting a duplicate gel into 5 mm-thick slices and extracting each piece with 1.0 ml 25 mM KCl.

Bio-Sil SEC-400 chromatography

The native molecular mass of the purified extracellular trehalase was estimated using a Bio-Sil SEC-400 HPLC gel filtration column (Bio-Rad, USA). A sample of the purified enzyme was injected onto a column (300×7.8 mm) equilibrated and eluted with 100 mM Hepes buffer (pH 6.8), containing 150 mM NaCl and 10 mM sodium azide. The molecular mass markers were tyroglobulin (670 kDa), apoferritin (445 kDa), bovine gama globulin (158 kDa), ovalbumin (45 kDa), and equine myoglobin (17 kDa). The column was eluted at 25°C at a flow rate of 1 ml/min.

Determination of neutral carbohydrates and proteins

The total neutral carbohydrates were estimated by the phenol sulfuric method of Dubois *et al*. (1956) using glucose as a standard. The protein concentration was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Studies of thermal stability

Studies of thermal inactivation were performed by incubating aliquots of the purified enzyme diluted either in 50 mM sodium acetate buffer (pH 5.0), or 1 mM trehalose in water at different temperatures and for different time intervals. After heating, the samples were maintained in crushed ice until being assayed for residual activity.

When trehalose was tested as a protecting agent, the mixture was precipitated with 3 volumes of ethanol (95%), centrifuged at 11,000×g for 15 min, and the enzyme in the pellet was dissolved in water and assayed for its residual activity. A control for the effect of ethanol precipitation on the enzyme activity was performed using a sample of the purified enzyme that was not submitted to heat treatment.

Table 1. Effect of carbon source on the extracellular trehalase production by *M. pulchella*

Carbon source (1%)	Total units (U)	Total protein (mg)	Specific activity (U/mg protein)
Deficient[a]	1.20±0.14	0.90±0.13	1.33±0.20
Glucose	15.03±1.20	9.53±1.14	1.58±0.13
Trehalose	12.82±0.98	9.81±0.88	1.31±0.12
Maltose	21.05±1.26	15.02±0.90	1.40±0.12
Starch	18.36±1.65	6.50±0.98	2.82±0.23
Sucrose	7.89±1.18	7.33±0.88	1.08±0.15
Xylose	16.57±1.49	17.39±1.22	0.95±0.13
Arabinose	16.03±1.28	12.32±1.49	1.30±0.14
Glycerol	10.55±1.26	5.57±0.95	1.89±0.26
Fructose	14.01±1.44	10.59±1.75	1.32±0.16
Mannose	14.83±1.49	10.39±1.25	1.43±0.17

[a] Deficient contains only low amount of carbohydrate present in yeast extract. Data are the Means±SD of at least three different cultures grown at 40°C for 96 h.

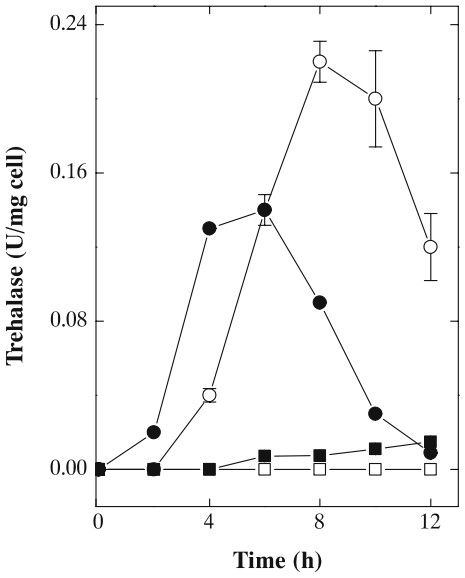

Fig. 1. Time-course of acid extracellular trehalase production by *M. pulchella.* Humid mycelium (300 mg) pre-grown in 1% glucose was reincubated in 50 ml culture medium containing 1% glucose (□); 1% starch (○); 1% trehalose (●) or no carbon source (■).

Results

Effect of carbon source on the extracellular trehalase production

Although several sugars were tested for extracellular trehalase production by *M. pulchella*, high levels of enzymatic activity were obtained with glucose, maltose, starch, xylose and arabinose as the main carbon sources (Table 1). In contrast, only moderate levels of total enzyme units were secreted into the culture medium in the presence of trehalose, the natural substrate of the enzyme. Although slightly less enzyme units were produced in starch, compared to maltose, it was chosen as a carbon source for mold growth due to the higher enzymatic specific activity obtained (Table 1).

Figure 1 demonstrates the time-course of extracellular trehalase production in glucose-, starch- and trehalose-reincubated cultures. In starch, the extracellular enzyme activity in-

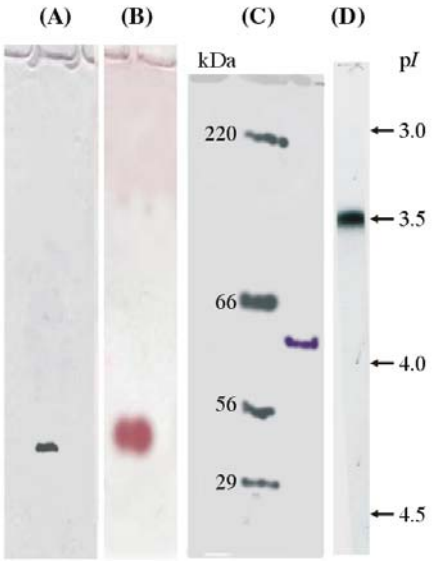

Fig. 2. PAGE and SDS-PAGE of the purified extracellular trehalase from *M. pulchella* var. sulfurea. Non-denaturing electrophoresis was carried out in 6% acrylamide gels, at pH 8.9. In denaturing conditions, 7% acrylamide gels were used. Each lane contained 20 μg of purified enzyme. Lanes: A, native conditions, stained with Coomassie blue; B, native conditions, stained for activity; C, denaturing conditions; D, Isoelectric focusing, stained with Coomassie blue.

Table 2. Purification of the extracellular trehalase from *M. pulchella*

Step	Total protein (mg)	Total units (U)	Specific activity (U/mg Prot)	Yield (%)	Purification (-fold)
Crude filtrate	314.0	780.0	2.5	100.0	1.0
DEAE-cellulose pH 7.0	14.0	119.0	8.5	15.3	3.4
Phenyl-Sepharose	1.7	19.5	11.5	2.5	4.6
DEAE-cellulose pH 5.0	0.6	49.0	81.7	6.3	32.7

Data are the means of six different preparations.

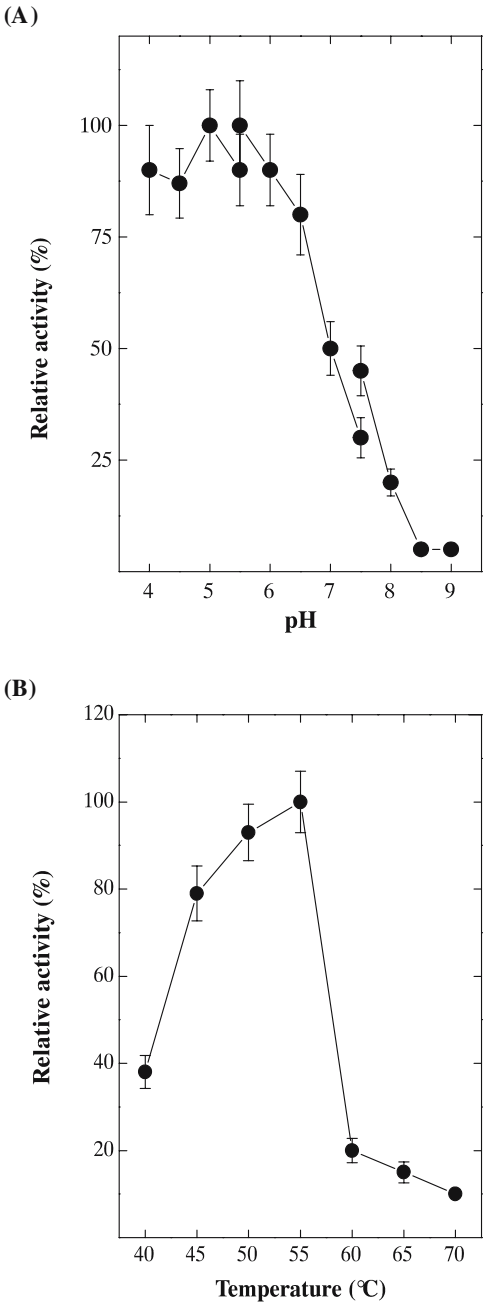

Fig. 3. Effect of pH (A) and temperature (B) on trehalase activity. The buffers used, at 50 mM concentration, were sodium acetate (pH 4.0-5.5), MES (pH 5.5-7.5), and Tris-HCl (7.5-9.0). The effect of temperature was assayed in 50 mM sodium acetate buffer at pH 5.0.

creased after 4 h, and reached the maximum level at 8 h, while in trehalose, the enzyme production was promptly detected after 2 h reincubation, attaining maximum levels between 4 to 6 h growth. However, a fast decrease of enzyme levels was observed in the trehalose-reincubated cultures after 6 h, while levels of about 50% of maximal activity were detected after 12 h in starch. Extracellular trehalase activity was not detected in glucose-reincubated cultures until 12 h growth.

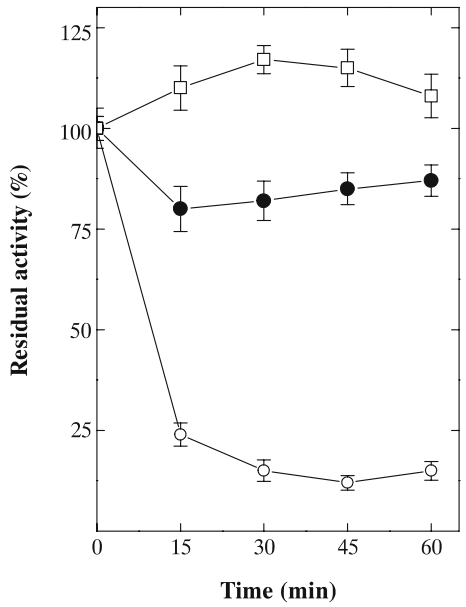

Fig. 4. Thermal stability of the purified trehalase from *M. pulchella*. The enzyme was incubated in 50 mM sodium acetate buffer (pH 5.0) at 60℃ (●) and 65℃ (○), or in buffer containing 1 mM trehalose at 65℃ (□).

Purification and molecular properties of the extracellular trehalase

Table 2 summarizes the typical results of a purification protocol for extracellular *Malbranchea* trehalase. After the last chromatographic step, the specific activity of the purified enzyme

Table 3. Effect of potential inhibitors and activators on trehalase activity

Addition (1 mM)	Relative activity (%)
Control	100±4
NaCl	98±5
$AgNO_3$	110±2
$CaCl_2$	58±4
$MgCl_2$	97±4
$MnCl_2$	170±2
KCl	97±5
NH_4Cl	94±4
$AlCl_3$	57±7
$CuCl_2$	90±9
$ZnCl_2$	92±6
$CoCl_2$	114±4
$Pb(C_2H_3O_2)_2$	66±8
Fe SO_4	39±4
$HgCl_2$	44±6
$BaCl_2$	98±4
ß-Mercaptoethanol	98±4
EDTA	94±5
ADP	97±4
ATP	99±5
ADP (10 mM)	94±6
ATP (10 mM)	96±4

Data are the Means±SD of four different enzymatic assays

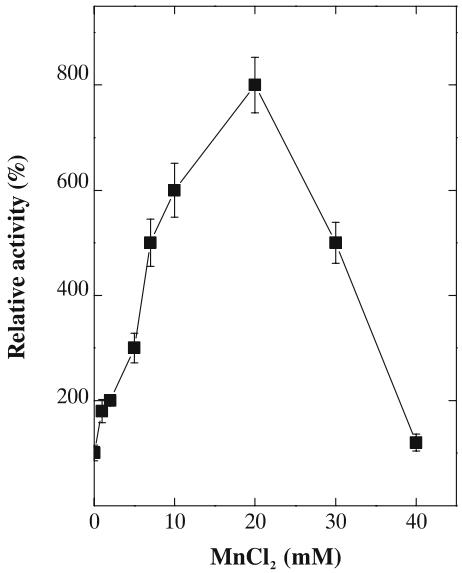

Fig. 5. Effect of $MnCl_2$ on the activity of the extracellular trehalase purified from *M. pulchella*. The specific activity value of the control (without $MnCl_2$) was 85 U/mg prot.

was 81.7 U/mg protein, with a purification of about 33-fold. Non-denaturing 6% PAGE of the purified enzyme run at pH 8.9 showed a single protein band after Coomassie Brilliant blue staining (Fig. 2A). A duplicate gel showed that the band stained for trehalase activity was coincident with the protein band (Fig. 2B). Analysis of the purified enzyme by SDS-PAGE also showed a single band (stained with Coomassie bue) corresponding to a molecular mass of about 52 kDa (Fig. 2C).

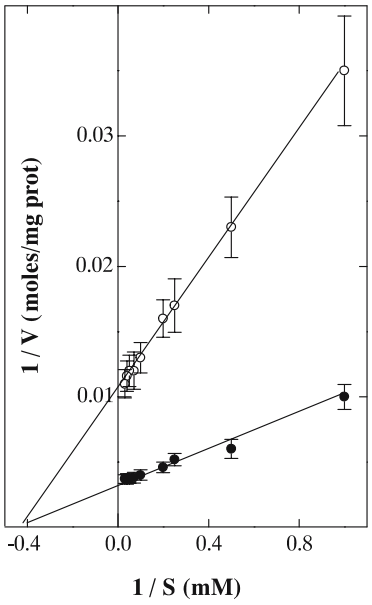

Fig. 6. Lineweaver-Burk plots for determination of the kinetic parameters (K_M and V_{Max}) for trehalose hydrolysis by the extracellular trehalase purified from *M. pulchella*.
Symbols: (○) without $MnCl_2$; (●) with 2 mM $MnCl_2$.

Table 4. Kinetic constants for trehalose hydrolysis by *M. pulchella* extracellular trehalase

Conditions	K_M (mM)	V_{max} (U/mg protein)	Catalytic efficiency (Vmax/KM)
Control	2.70±0.29	90.06±10.81	33.36±4.00
$MnCl_2$ (2 mM)	2.67±0.18	360.15±21.61	134.89±8.09
$MnCl_2$ (10 mM)	2.58±0.13	792.24±47.53	307.07±15.35

Data are the Means±SD of at least four different assays

Molecular sieving of the purified enzyme in a Bio-Sil Sec 400 column resulted in an apparent molecular mass of 104 kDa. The carbohydrate content of the purified enzyme was estimated to be 19% and its isoelectric point was 3.5 (Fig. 2D).

Effect of pH and temperature

The purified trehalase showed maximal activity at a pH range from 5.0 to 5.5 (Fig. 3A), and the activity decreased just around 15% down to pH 4.0. However, at pH 7.0 and 8.0 it corresponded to about 50 and 20% of the maximum, respectively. The temperature optimum for the trehalase activity was 55°C (Fig. 3B), and at 50°C the enzyme presented about 90% of its maximal activity. However, an abrupt decrease occurred above 55°C, and the activity estimated at 60°C corresponded to only 20% of the maximum.

Thermal stability studies revealed that *Malbranchea* trehalase has good resistance to heat, loosing no more than 20% of its activity when incubated at 60°C for up to 60 min in 50 mM sodium acetate, pH 5.0. However, a residual activity of only 22% was estimated after 15 min of incubation at 65°C (Fig. 4). Trehalose, at 1 mM concentration, completely protected the enzyme up to 1 h against inactivation even at 65°C (Fig. 3).

Effect of potential inhibitors and activators

The cations Na^+, Mg^{2+}, K^+, NH_4^+, Cu^{2+}, Zn^{2+}, Co^{2+}, or Ba^{2+}, at 1.0 mM concentration, did not affect the enzymatic activity. In contrast, 42, 43, 34, 61, and 66% inhibition was observed for Ca^{2+}, Al^{3+}, Pb^{2+}, Fe^{2+}, and Hg^{2+}, respectively (Table 3). Surprisingly, Ag^+ did not inhibit the trehalase activity. Manganese ions strongly stimulated the activity of the purified enzyme, reaching an 8-fold activation at 20 mM concentration (Fig. 5). Moreover, at 30 mM Mn^{2+} the enzymatic activity was yet 5-fold higher than that observed in the absence of metallic ions. EDTA and ß-mercatoethanol had no noteworthy effect on the enzymatic activity (Table 3). Remarkably, ADP or ATP, even at 10 mM concentration, did not inhibit the purified *Malbranchea* extracellular trehalase.

Enzyme specificity and kinetic parameters

The purified *Malbranchea* extracellular trehalase was highly specific for trehalose as a substrate and has not hydrolyzed cellobiose, lactose, maltose, raffinose or sucrose, at 10 mM concentration. Comparative studies were performed to determine the kinetic parameters K_M and V_{max} for trehalose hydrolysis by *Malbranchea* trehalase in the presence or absence of manganese ions. Although similar K_M values were measured under both conditions (Fig. 6), V_{max} and catalytic efficiency were 8.8 and 9.2-fold higher, respectively, in the presence of 10 mM $MnCl_2$ (Table 4).

Discussion

Data presented in the present study support the hypothesis of the occurrence of trehalases with mixed properties in thermophilic fungi, as suggested for the first time by Jorge *et al.* (1997). Recently, mixed features of neutral and acid trehalases were also demonstrated in *C. albicans* (Sánches-Fresneda *et al*., 2009).

Usually, high production of trehalases by thermophilic fungi occurs in media containing mannitol (Cardello *et al*., 1994) or starch (Almeida *et al*., 1999), but not when the molds are grown in glucose as a carbon source. Here, high levels of trehalase production by aged cultures of *Malbranchea* were observed using glucose as the main carbon source, although the enzyme was detected in the culture medium only after a complete consumption of glucose (data not shown). Currently, the physiological functions of thermophilic fungi mixed trehalases (Zimmerman *et al*., 1990; Cardello *et al*., 1994; Kadowaki *et al*., 1996) are not fully understood. However, the present studies with young cultures suggest that the acid extracellular *Malbranchea* trehalase production may be controlled by carbon regulatory catabolic circuits, since enzyme production was strongly inhibited by the presence of glucose in the culture medium, and stimulated by trehalose and starch. Moreover, in the presence of trehalose, traces of trehalase appeared just 2 h after reincubation, while in cultures containing starch the enzyme was detected only after 4 h reincubation. These data suggest that the main role of the extracellular trehalase from *Malbranchea* may be the utilization of trehalose as a carbon source. Thus, the present study demonstrates for the first time a role for the mixed trehalases produced by thermophilic fungi. Remarkably, starch is a good inducer of the extracellular trehalase from *Malbranchea*. A reasonable supposition to explain this fact may be the production of α-glucosidase, which converts maltooligosaccharides in trehalose, by *M. pulchella*, as known for *Chaetomium thermophilum* var. coprophilum, (Giannesi *et al*., 2006). Hence, if this speculation is valid, trehalose may be the true inducer of *Malbranchea* extracellular trehalase when the mold is grown in starch. On the other hand, an α-glucosidase from another thermophilic mold, *Thermoascus aurantiacus*, hydrolyzes trehalose (Carvalho *et al*., 2010), thereby suggesting some relationship between the mobilization of trehalose and maltooligosacharides in thermophilic fungi.

Analysis of the purified trehalase molecular mass by SDS-PAGE and gel filtration suggested that the enzyme is a dimer. Most of the purified trehalases exhibit molecular masses above 100 kDa (Jorge *et al*., 1997). In fact, an oligomeric nature was observed for all thermophilic fungi trehalases purified to date (Zimmerman *et al*., 1990; Cardello *et al*., 1994; Kadowaki *et al*., 1996; Almeida *et al*., 1999). The pI value of 3.5 determined for *Malbranchea* extracellular trehalase was quite similar to those found for trehalases purified from other thermophilic fungi (Cardello *et al*., 1994; Kadowaki *et al*., 1996). *Malbranchea* extracellular trehalase exhibited 19% of carbohydrates, a value unexpectedly close to that found for a cytosolic trehalase purified from *Humicola grisea* var. thermoidea (Cardello *et al*., 1994). In contrast, two extracellular trehalases from *H. grisea* var. thermoidea and *Scytalidium thermophilum* exhibited 56% and 81% sugar contents, respectively (Zimermmann *et al*., 1990; Kadowaki *et al*., 1996). The optimum pH of *Malbranchea* extracellular trehalase was 5.0 to 5.5, similar to those reported for other acid trehalases, either from mesophilic and thermophilic fungi, lying in the range from 4.0 to 6.5 (Jorge *et al*., 1997). However, the pH profile obtained suggested that *Malbranchea* enzyme is more active at pH levels below the optimum than other acid trehalases from thermophilic fungi (Zimmerman *et al*., 1990; Cardello *et al*., 1994; Kadowaki *et al*., 1996; Almeida *et al*., 1999).

The optimum temperature for extracellular *Malbranchea* trehalase activity was 55°C, similarly as observed for several acid trehalases from mesophilic and thermophilic fungi (Jorge *et al*., 1997). However, the enzyme from *Malbranchea* exhibited only about 20% of its maximal activity at 60°C. Aggregation of glycoproteins induced by heat may be accelerated by glycosylation (Broersen *et al*., 2007), and thermophilic fungi trehalases are glycosylated and tend to aggregate (Cardello *et al*., 1994; Kadowaki *et al*., 1996). Thus, the abrupt drop of *Malbranchea* trehalase activity at 60°C may be attributed to the formation of enzyme aggregates in the reaction medium. However, the good thermal stability of the enzyme in buffer at 60°C suggests that *Malbranchea* trehalase may be reversible disaggregated when maintained in an ice bath before the residual activity assay, thereby explaining the maintenance of about 75-80% of control activity after 60 min incubation. This hypothesis is reinforced by the fact that protein refolding may also be stimulated by glycosylation (Wang *et al*., 1996). Thus, enzyme-bound carbohydrates may act simultaneously as agents of protein aggregation, at elevated temperatures (Broersen *et al*., 2007) or refolding, at low temperatures (Wang *et al*., 1996).

Trehalases purified from thermophilic sources are usually activated by calcium, manganese and cobalt ions (Jorge *et al*., 1997; Lucio-Eterovic *et al*., 2005). Surprisingly, even though the acid trehalase from *Malbranchea* was strongly activated by manganese, it was inhibited by calcium and insensitive to cobalt. Moreover, unlike other trehalases purified from thermophilic fungi, *Malbranchea* trehalase was insensitive to silver ions. ATP and ADP were also reported to be inhibitors of thermophilic fungi trehalases (Zimmerman *et al*., 1990; Cardello *et al*., 1994; Kadowaki *et al*., 1996), contrasting with the insensitivity of *Malbranchea* trehalase to these nucleotides. Like other trehalases, the extracellular enzyme from *Malbranchea* was strictly specific for trehalose, and the K_M for the substrate hydrolysis was similar to those reported for other thermophilic fungi trehalases, in the range 2.3-3.6 mM (Jorge *et al*., 1997), and also for enzymes from mesophilic organisms, in the range 0.2-20 mM (Elbein, 1974; Jorge *et al*., 1997). Manganese ions did not affect the K_M value of *Malbranchea* trehalase for the substrate hydrolysis, but strongly increased the value of V_{max} and consequently, the catalytic efficiency. In contrast, manganese or calcium ions induced pronounced changes both in K_M and V_{max} values for trehalose hydrolysis by trehalases from *S. thermophilum* (Kadowaki *et al*., 1996), while only the Vmax values were affected by manganese ions, considering the trehalases purified from *H. grisea* var. thermoidea (Zimmemann *et al*., 1990; Cardello *et al*., 1994). In comparison to mesophilic fungi trehalases, those from thermophilic fungi exhibited considerably higher values of V_{max}, and consequently higher catalytic efficiencies, defined as the ratios of V_{max} and K_M values. The elevated specific activity values (84.6±9.3 U/mg, 736±74

U/mg, non-activated and activated by manganese, respectively) exhibited by the purified *Malbranchea* acid extracellular trehalase are similar to those found for *Humicola* trehalases, used to quantitatively estimate trehalose in biological samples (Neves *et al*., 1994). Altogether, the results reported herein strongly reinforce that thermophilic fungi produce a type of trehalase, different from the conventional acid and neutral enzymes. Moreover, the biochemical characterization of *Malbranchea* enzyme suggests that even amongst thermophilic fungi, the trehalases may show divergent properties, particularly concerning the response to activators and inhibitors. Thus, the properties of thermophilic fungi trehalases deserve further investigation, aiming the conception of a classification system that may aid to efficiently distinguish all trehalase types and, together with molecular structure data, give some idea of their evolutionary origin.

Acknowledgements

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). M.G.P. received a MSc scholarship from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). H.F.T., J.A.J., M.L.T.M.P., and R.P.M.F. are research fellows of CNPq. We thank Mauricio de Oliveira for technical assistance.

References

Almeida, E.M., M.L.T.M. Polizeli, H.F. Terenzi, and J.A. Jorge. 1999. Biochemical characterization of a Ca^{2+}-dependent acid trehalase activity from the thermophilic fungus *Chaetomium thermophilum* var. coprophilum. *FEMS Microbiol. Lett*. 171, 11-15.

Bergmeyer, H.U. and E. Bernt. 1974. Methods of enzymatic analysis, vol. 3, pp. 1205-1215. *In* H.U. Bergmeyer (ed.), Verlag-Chimie/ Academic Press, New York, NY, USA.

Bonini, B.M., P. Van Dijck, and J.M. Thevelein. 2004. Trehalose metabolism enzymatic pathways and physiological fuctions, pp. 291-332. *In* R. Brambl and G.A. Marzluf (eds.), The Mycota III Biochemistry and Molecular Biology, 2nd ed. Springer Verlag, Berlin, Germany.

Broersen, K., M. Elshof, J. de Groot, A.G.J. Voragen, R.J. Hamer, and H.H.J. de Jongh. 2007. Aggregation of ß-lactoglobulin regulated by glucosylation. *J. Agric. Food Chem*. 55, 2431-2437.

Cardello, L., H.F. Terenzi, and J.A. Jorge. 1994. A cytosolic trehalase from the thermophilic fungus *Humicola grisea* var. thermoidea. *Microbiology* 140, 1671-1677.

Carvalho, A.F., M. Bosocolo, R. Silva, H. Ferreira, and E. Gomes. 2010. Purification and characterization of the α-glucosidase produced by thermophilic fungus *Thermoascus aurantiacus* CBMAI 756. *J. Microbiol*. 48, 452-459.

Davis, B.J. 1964. Disc electrophoresis II - Method and application to human serum proteins. *Ann. NY Acad. Sci*. 121, 404-427.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem*. 28, 350-356.

Elbein, A.D. 1974. The metabolism of α, α-trehalose. *Adv. Carbohydr. Chem. Biochem*. 30, 227-256.

Giannesi, G.C., M.L.T.M. Polizeli, H.F. Terenzi, and J.A. Jorge. 2006. A novel α-glucosidase from *Chaetomium thermophilum* var. *coprophilum* that converts maltose into trehalose: Purification and partial characterization of the enzyme. *Proc. Biochem*. 41, 1729-1735.

Jorge, J.A., M.L.T.M. Polizeli, J.M. Thevelein, and H.F. Terenzi. 1997. Trehalases and trehalose hydrolysis in fungi. *FEMS Microbiol. Lett*. 154, 165-171.

Kadowaki, M.K., M.L.T.M. Polizeli, H.F. Terenzi, and J.A. Jorge. 1996. Characterization of trehalase activities from the thermophilic fungus *Scytalidium thermophilum*. *Biochim. Biophys. Acta* 1291, 199-205.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 222, 680-685.

Leone, F.A., L. Degreve, and J.A. Baranauskas. 1992. Sigraf-a versatile computer program for fitting enzyme kinetic data. *Biochem. Educ*. 20, 94-96.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randal. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem*. 193, 265-275.

Lúcio-Eterovic, A.K.B., J.A. Jorge, M.L.T.M. Polizeli, and H.F. Terenzi. 2005. Biochemical characterization of the trehalases of thermophilic fungi: An enzyme with mixed properties of neutral and acid trehalase. *Biochim. Biophys. Acta* 1723, 201-207.

Miller, A. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*. 31, 426-428.

Neves. M.J., H.F. Terenzi, F.A. Leone, and J.A. Jorge. 1994. Quantification of trehalose in biological samples with a conidial trehalase from the thermophilic fungus *Humicola grisea* var. thermoidea. *Word J. Microbiol*. 10, 17-19.

O'Farrel, P.Z., H.M. Goodman, and P.H. O'Farrel. 1977. High resolution two dimensional electrophoresis of basic as well as acidic proteins. *Cell* 12, 1133-1142.

Panek, A.D. 1969. Adenosine triphosphate inhibiton of yeast trehalase. *J. Bacteriol.* 99, 904-905.

Parrow, J.L., M. Jules, G. Beltran, and J. François. 2005. Acid trehalase in yeasts and filamentous fungi: localization, regulation and physiological function. *FEMS Yeast Res.* 5, 503-511.

Sánchez-Fresneda, R., P. González-Párraga, O. Esteban, L. Laforet, E.Valentín, and J.C. Argüélles. 2009. On the biochemical classification of yeast trehalases: *Candida albicans* contains two enzymes with mixed features of neutral and acid trehalase activities. *Biochem. Biophys. Res. Comm*. 383, 98-102.

Schick, I., D. Haltrich, and K.D. Kulbe. 1995. Trehalose phosphorylase from *Pichia fermentans* and its role in metabolism of trehalose. *Appl. Microbiol. Biotechnol*. 43, 1088-1095.

Simola, M., A.L. Stranius, and M. Makarow. 2000. Trehalose is required for conformational repair of heat-denatured proteins in the yeast endoplasmic reticulum but not for maintenance of membrane traffic functions after severe heat stress. *Mol. Microbiol*. 37, 4-53.

Singer, M.A. and S. Lindquist. 1998. Thermotolerance in *Saccharomyces cerevisiae*: the yin and yang of trehalose. *Trends Biotechnol.* 16, 460-468.

Thevelein, J.M. 1984. Regulation of trehalose mobilization in fungi. *Microbiol. Rev*. 48, 42-59.

Thevelein, J.M. 1988. Topical review: regulation of trehalase activity by phosphorylation - dephosphorylation during developmental transitions in fungi. *Exp. Mycol*. 12, 1-12.

Wang, C., M. Eufemi, C. Turano, and A. Giartosio. 1996. Influence of carbohydrate moiety on the stability of glycoproteins. *Biochem*. 35, 7299-7307.

Zimmerman, A.L.S., H.F. Terenzi, and J.A. Jorge. 1990. Purification and properties of an extracellular conidial trehalase from *Humicola grisea* var. thermoidea. *Biochim. Biophys. Acta* 1036, 41-46.