Characterisation of an acid trehalase produced by the therмотolerant fungus \textit{Rhizopus microsporus} var. \textit{rhizopodiformis}: Biochemical properties and immunochemical localisation

Ana Carla Medeiros Morato de Aquino, Simone Carvalho Peixoto-Nogueira, João Atílio Jorge, Héctor Francisco Terenzi, Maria de Lourdes Teixeira de Moraes Polizeli *

Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, 14.040-901 Ribeirão Preto, SP, Brazil

Received 11 May 2005; received in revised form 28 July 2005; accepted 29 July 2005

First published online 22 August 2005

Edited by M. Moracci

Abstract

An acid trehalase from \textit{Rhizopus microsporus} var. \textit{rhizopodiformis} was purified to apparent homogeneity. The molecular weight by SDS–PAGE (60 kDa) or Sephacryl S-200 filtration (105 kDa) suggested a homodimer. The carbohydrate content was 72%. Endoglycosidase H digestion resulted in one sharp band of 51.5 kDa in SDS–PAGE. pH and temperature optima were 4.5 and 45 °C, respectively. The isoelectric point was 6.69 and activation energy was 1.14 kcal mol$^{-1}$. The enzyme was stable for 1 h at 50 °C and decayed at 60 °C (t$_{50}$ of 1.3 min.). Apparent $K_M$ for trealose was 0.2 mM. Immunolocalisation studies showed the enzyme tightly packed at the surface of the cells.

Keywords: Trehalose; Fungi; Exoenzymes; $\alpha,\alpha$-Trehalase; \textit{Rhizopus microsporus}; Immunochemical localisation

1. Introduction

Trehalose is a disaccharide found in a variety of organism, including bacteria, yeasts, filamentous fungi, plants and insects [1]. The enzyme trehalase (EC 3.2.1.28) specifically hydrolyses this carbohydrate to glucose. Fungal trehalases were grouped into two categories according to their pH: acid and neutral trehalases, which display maximal activity around pH 4.5 and 7.0, respectively. Despite showing a strict specificity for trehalose, these two types of trehalases are completely different in terms of primary structure, subcellular localisation, and biochemical and regulatory properties [2–4].

The biological role of neutral trehalases is related to the mobilisation of endogenous cytosolic trehalose, under the control of developmental programs, chemical and nutrient signals, or stressful stimuli. On the other hand, acid trehalases, mainly extra-cytosolic (extracellular or vacuolar) glycoproteins, enable the organism to utilise exogenous trehalose as a carbon source. Many fungi and yeasts can use trehalose as an extracellular
source of carbon [3]. Some studies indicate that for *Saccharomyces cerevisiae* this disaccharide could be acquired by direct uptake, where trehalose could reach the vacuole by an endocytotic process and be degraded by the vacuolar acid trehalase [5,6]. More recent evidence, however, shows that in this yeast acid trehalase is extracellular rather than vacuolar and, therefore, the hydrolysis of exogenous trehalose would occur mainly in the periplasmic space [7]. This also seems to be the case for filamentous fungi which hydrolyse extracellular trehalose using extracellular acid trehalases. These enzymes may be localised on the cell wall, periplasmic space [7–10] or secreted into the culture medium [11–13]. Fungal acid trehalases have been well characterised from a biochemical standpoint in ascomycetes, hemiascomycetes, and some thermophilic deuteromycetes. Acid trehalases from ascomycetes and hemiascomycetes are different from those from thermophilic deuteromycetes. Acid trehalases from ascomycetes and hemiascomycetes are indeed con-
different from those from thermophilic deuteromycetes, in the sense that the latter enzymes show mixed properties of acid and neutral trehalases [3].

Until quite recently it was suspected that zygomycetes, such as *Mucor rouxii* or *Phycomyces blakesleeanus* would only possess the neutral type of trehalase, and not the acid type of the enzyme [14]. Later on, a study from our laboratory reported that *M. rouxii* indeed contained the two types of trehalases [13]. This was the first report of the existence of acid trehalase in a Zygomyecete. We decided to better characterise the acid trehalase from zygomycetes, in order to know whether this enzyme was similar to the acid trehalases of ascomycetes or hemiascomycetes. In the present study we selected the acid trehalase from *Rhizopus microsporus* var. *rhizopodiformis*, a thermotolerant zygomyecete, which produced large amounts of enzyme. This acid trehalase was purified and biochemically characterised.

2. Materials and methods

2.1. Organism and growth conditions

*R. microsporus* var. *rhizopodiformis* was isolated in our laboratory from soil of the Brazilian “cerrado” (Pirassumunga, São Paulo, Brazil) and identified by André Tosello Foundation (Campinas, São Paulo, Brazil), as described by Schipper and Stalpers [15]. The fungus was maintained at 40 °C, in slants of solid 4% oatmeal baby food (Quaker) medium. Spores (3 × 10^7 ml^{-1}) obtained from 10-day-old cultures were inoculated into 250 ml Erlenmeyer flasks containing 50 ml minimal Vogel liquid medium [16], pH 6.0, using 1% trehalose (Sigma™) as carbon source. The cultures were incubated for 48 h at 40 °C, 100 rev min^{-1}. Mycelial pads were harvested by filtration, blotted on filter paper, and stored at −15 °C until use. The culture filtrates were saved as a source of crude extracellular enzyme.

2.2. Enzymatic assays and protein determination

Acid trehalase activity was determined by measuring the glucose released by the glucose oxidase/peroxidase procedure [17]. The reaction system was constituted of 50 μl of the diluted enzyme, 100 μl of 75 mM trehalose and 100 μl of 50 mM sodium acetate buffer, pH 4.5. The samples were incubated for 20 min at 37 °C, boiled for 5 min and the glucose released was quantified. An enzyme unit is the amount of enzyme that produces 1 μmol of glucose per minute. Protein was estimated as described by Lowry et al. [18] using bovine serum albumin as standard.

2.3. Enzyme purification

All steps were carried out at 4 °C. The culture filtrate was dialysed against 10 mM sodium acetate buffer, pH 4.5 and applied to a DEAE-cellulose (diethylaminoethyl cellulose) column (2.0 × 12.7 cm) equilibrated with the same buffer and eluted with 80 ml of a linear concentration gradient (0–1.0 M) of sodium chloride dissolved in the acetate buffer. Fractions of 0.6 ml were collected at a flow rate of 2 ml min^{-1}. Fractions showing acid trehalase activity were pooled, and applied to a filter device (Millipore™), with a 30 kDa cut-off membrane, to concentrate and desalt the sample. The acid trehalase obtained was utilised for biochemical characterisation.

2.4. Electrophoresis and isoelectric focusing

Pure trehalase preparation were submitted to electrophoresis [19] in 10% SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and stained with silver solution [20]. Non-denaturant 7% polyacrylamide gel (PAGE) was carried out according to Davis [21] and stained with silver solution. Molecular weight standards used were: β-galactosidase (116 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). Acid trehalase was visualised in 7% PAGE gels using glucose oxidase (Merck™), 30U per gel, nitroblue tetrazolium (Sigma™), 0.4 g l^{-1} and phenazine methosulfate (Sigma™), 0.2 g l^{-1}. The gel was incubated until the activity band was visualised. The isoelectric focusing (pI) had been developed according to O’Farrel et al. [22]. It was used a 5% polyacrylamide gel polymerised in tubes (0.5 × 15 cm) and added by Pharmalite™ ampholite (pH 3–10). The protein was visualized with 0.05% coomassie brilliant blue R-200 dissolved in a solution composed by 90.8 ml of 50% methanol and 9.2 ml of acetic acid.

2.5. Gel filtration

The molecular weight of the purified acid trehalase was estimated by Sephacryl S-200 gel filtration.
A sample of enzyme was loaded to the column (1.8 × 122 cm), equilibrated and eluted with 50 mM acetate buffer, pH 4.5 plus 150 mM NaCl. Fractions of 1 ml were collected at a flow rate of 12 ml h−1. The column was previously equilibrated with the molecular weight standards: aldolase (158 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa).

2.6. Carbohydrate content and deglycosylation of the acid trehalase protein

Total neutral carbohydrate was estimated by the phenol–sulphuric acid method [23] using mannose as a standard. Deglycosylation of the protein was carried out using Endoglycosidase H (Boehringer–Mannheim). The reaction system was constituted by the purified enzyme 100 μl (0.15 μg protein), 14 M β-mercaptoethanol 0.4 μl, 1% SDS 5 μl, 500 mM sodium acetate buffer, pH 5.0, 10 μl, and Endoglycosidase H 5 mU. This preparation was incubated for 24 h at 37 °C, and then dialysed for 2 h against Milli Q water.

2.7. Determination of kinetic parameters

Maximum velocity (V_M) and apparent Michaelis–Menten constant (K_M), for trehalase hydrolysis were calculated according to Lineweaver–Burk [24] using the Origin 6.1 software. These constants were calculated from at least three (N ≥ 3) different preparations with consistent values.

2.8. Antibodies production and immunohistochemical localisation

The primary antibody (anti-acid trehalase) was obtained from New Zealand rabbits immunised with a sample of purified of acid trehalase (50 μg ml−1 for each immunisation). First, the sample was diluted 1:1 with complete Freund Adjuvant and applied in a New Zealand rabbit. After 21 days, a new sample was diluted 1:1 with incomplete Freund Adjuvant and applied again in the same animal. After 21 days, the immunisation was carried out with purified acid trehalase diluted 1:1 in saline solution. After 7 days blood was collected through of cardiac puncture. The serum was precipitated with saturated ammonium sulphate, resuspended in sodium phosphate buffer, 0.02 M, pH 7.5, and dialysed in the same buffer. The sample was eluted in DEAE-cellulose column and the fraction correspondent to IgG (did not bind the resin) was pooled. This fraction was resuspended in PBS buffer (NaCl 0.8%, KCl 0.02%, NaHPO_4 0.144%, KH_2PO_4 0.024%, pH 7.4) added of 0.1% BSA and filtrated through Nalgene 0.45 μm filter.

Acid trehalase was detected “in situ” using mouse anti-trehalase antibodies [25]. For immunofluorescence assays were used 4-h-old germlings obtained from liquid cultures supplemented with glucose or trehalose. These cells were washed twice in PBS buffer (NaCl 1.38 M, KCl 27 mM, KH_2PO_4 15 mM, Na_2HPO_4 32 mM, distilled H_2O sufficient to 1000 ml, pH 7.2–7.4), and embedded in tissue freezing medium TFS-402 (EM-sciences). Ten-micron cryosections were washed for 5 min in PBS, PBS with 100 mM glycine and PBS + 1% BSA. Sections were then incubated with primary antibody anti-acid trehalase diluted at 1:10 in PBS + 1% BSA, for 1 h, at room temperature. After that, samples were washed on PBS (6 times for 5 min each one) and then incubated for 30 min with secondary antibody IgG anti-rabbit conjugated with ALEXA 488 (Mol Probes, Ont., Canada), at room temperature [26]. Finally, the samples were washed 8 times with PBS, one time on distillate water and cover slips mounted with Fluoromount G (EM Sciences). Controls consisted of sections incubated without primary antibody. The samples were observed in a photographed using an Olympus BX 50 fluorescence microscope.

3. Results and discussion

3.1. Acid trehalase purification

The acid trehalase from *R. microsporus* was purified to apparent electrophoretic homogeneity in just two steps. First, the filtrate (500 ml) from a 48-h-old culture, dialysed against 10 mM sodium acetate buffer, pH 4.5, was applied to a DEAE-cellulose ion exchange chromatography column, and was eluted as described under Section 2. A sharp peak corresponding to acid trehalase activity was eluted with 0.35 M NaCl (Fig. 1). Second, the pool of active fractions was applied to a Millipore filter device, with a membrane of 30 kDa cut-off. The
acid trehalase activity was retained, while other proteins were eliminated. This acid trehalase preparation was used for biochemical characterisation. The results of a typical purification procedure are summarised in Table 1. The enzyme was purified 43-fold with 44% recuperation.

3.2. Molecular properties

The carbohydrate content of purified acid trehalase was estimated to be 72%. This glycoprotein migrated as a single band in 10% SDS–PAGE (Fig. 2A) with molecular weight of about 60 kDa. Digestion of the protein with Endoglycosidase H resulted in one distinct protein band of 51.5 kDa by SDS–PAGE (Fig. 2B), corresponding to the mannose-free acid trehalase moiety. In PAGE, only a protein band was revealed with silver nitrate (Fig. 2C), which was coincident with the band of activity in a gel run in parallel (Fig. 2D). The isoelectric point corresponded to 6.69. The same as for all acid trehalases described up to date this enzyme was a glycoprotein of high molecular weight. For instance, the native extracellular (Mₐ 370 kDa, 5 subunits 82 kDa) and intracellular (Mₐ 398 kDa, 5 subunits 85 kDa) acid trehalases from Scytalidium thermophilum have carbohydrate contents of 81% and 51%, respectively [12]. Acid trehalases from the thermophilic fungus Thermomyces lanuginosus (Mₐ 145 kDa) and of the mesophilic fungus Neurospora crassa (Mₐ 437 kDa, 4 subunits) are glycoproteins with 20% and 43% carbohydrate content, respectively [27].

Candida albicans genome data base was screened and the product of an open reading frame (IPF 19760/CA2574), with 41% identity to S. cerevisiae vacuolar acid trehalase (Ath1p), was identified and named Atc1p. The deduced amino acid sequence shows that Atc1p contains 20 potential sites for N-glycosylation [28]. R. microsporus acid trehalase was a highly glycosylated homodimer (72% carbohydrate), with Mₐ 105 kDa (gel filtration) and 60 kDa (SDS–PAGE). After deglycosylation by endoglycosidase H, the Mₐ corresponded to 51.5 kDa (SDS–PAGE). Similar results were reported for the vacuolar trehalase from Candida utilis which is a glycoprotein that can be precipitated by Con A-Sepharose. Treatment of this enzyme with endo H reduced its reactivity with the lectin without loss of enzyme activity [29].

3.3. Effect of temperature and pH

The effect of the temperature on the enzyme activity was determined in the range of 30–60 °C, in 50 mM sodium acetate buffer, pH 4.5. To determine the optimum of pH the activity assay was carried out at pH values between 3.5 and 5.5, in 50 mM sodium acetate buffer, at 37 °C. Optima of temperature and pH were 45 °C (Fig. 3A) and 4.5 (Fig. 3B), respectively. Thermal inactivation was determined by incubating the enzyme at 50 and 60 °C in the absence of substrate. The enzyme was relatively stable 1 h at 50 °C, but exhibited a half-life of 1.3 min at 60 °C (Fig. 3C). According to the Arrhenius plot, and of the data shown in Fig. 3A, the calculated activation energy was 1.14 kcal mol⁻¹.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg protein⁻¹)</th>
<th>Yield (%)</th>
<th>Purification factor (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude filtrate</td>
<td>32.87</td>
<td>9.77</td>
<td>0.29</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Dialysis</td>
<td>6.63</td>
<td>10.33</td>
<td>1.55</td>
<td>105.73</td>
<td>5.34</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>0.93</td>
<td>4.65</td>
<td>5.00</td>
<td>47.59</td>
<td>17.24</td>
</tr>
<tr>
<td>Millipore filter</td>
<td>0.33</td>
<td>4.19</td>
<td>12.69</td>
<td>42.88</td>
<td>43.76</td>
</tr>
</tbody>
</table>

U total = μmol min⁻¹.
3.4. Effect of metal ions and kinetic parameters

The effect of the chelating compound EDTA (ethylene diaminetetraacetic acid) and of some chlorines on the catalytic activity was investigated (Table 2). Acid trehalase activity from \textit{R. microsporus} was slightly increased (15–17\%) with Pb(C2H3O2)2. The addition of EDTA 1\% augmented the acid trehalase activity by 7\%. All other ions tested inhibited the trehalase activity. The more severe inhibitions were observed with 1 mM Fe2+ (17\%), 10 mM Al3+ (66\%), Mn2+ (15\%), Ba2+, Co2+ and Mg2+ (14\%). \(K_M\) and \(V_M\) values for trehalose as substrate corresponded to 0.2 mM and 45 nmol min\(^{-1}\) mg prot\(^{-1}\), respectively. The enzyme showed a typical Michaelian behaviour. These values are smaller than those previously reported for acid trehalases from \textit{S. thermophilum} (\(K_M\) 3.58 and 2.24 mM, to forms I and II, respectively) [12], \textit{Humicola grisea} (\(K_M\) 2.3 mM, forms I) [11] and (\(K_M\) 0.86 mM, form II) [30], \textit{T. lanuginosus} and \textit{N. crassa} (\(K_M\) 0.86 mM, form II) [30], \textit{baker's yeast} (\(K_M\) 1.4 mM) [31] and others [3], indicating a higher affinity for trehalose.

Table 2
Effect of metal ions and EDTA on the activity of the trehalase produced by \textit{R. microsporus} var. \textit{rhizopodiformis}

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM</td>
</tr>
<tr>
<td>No additions</td>
<td>100</td>
</tr>
<tr>
<td>AlCl(_3)</td>
<td>98.1</td>
</tr>
<tr>
<td>BaCl(_2)</td>
<td>97.4</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>103.4</td>
</tr>
<tr>
<td>CoCl(_2)-6H(_2)O</td>
<td>96.6</td>
</tr>
<tr>
<td>CuCl(_2)</td>
<td>98.3</td>
</tr>
<tr>
<td>EDTA</td>
<td>107.1</td>
</tr>
<tr>
<td>FeCl(_3)-6H(_2)O</td>
<td>82.6</td>
</tr>
<tr>
<td>HgCl(_2)</td>
<td>99.9</td>
</tr>
<tr>
<td>MgCl(_2)-6H(_2)O</td>
<td>97.6</td>
</tr>
<tr>
<td>MnCl(_2)-4H(_2)O</td>
<td>97.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>100.7</td>
</tr>
<tr>
<td>NH(_4)Cl</td>
<td>96.4</td>
</tr>
<tr>
<td>Pb(C(_2)H(_3)O(_2))(_2)</td>
<td>115.5</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>99.0</td>
</tr>
</tbody>
</table>

n.d., not detected.
β-glucosidase secretion diminishes. This explanation may be applied to R. microsporus suggesting that the presence of acid trehalase at the hyphal tip can be related to the secretion of the enzyme. In conclusion, we have shown that the Zygomycete fungus R. microsporus var. rhizopodiformis produced an acid trehalase with all the typical properties of similar enzymes produced by ascomycetes and hemiascomycetes.

Acknowledgements

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho de Desenvolvimento Científico e Tecnológico (CNPq). H.F.T., J.A.J., M.L.T.M.P. are Research Fellows of CNPq. A.C.M.A. and S.C.P.N. were recipient of FAPESP and CAPES Fellowships, respectively. This work was part of A.C.M.M.A. Ph.D. Thesis submitted to the Departamento de Biologia da Faculdade de Filosofia Ciências e Letras de Ribeirão Preto – USP. We thank Ricardo Alarcon and Mauricio Oliveira for technical assistance.

References


Fig. 4. Sections of Rhizopus microsporus var rhizopodiformis were immunolabeled for acid trehalase after 4 h of incubation in medium supplemented with 2% glucose (A,C) or 1% trehalose (B,D). Controls, without incubation of primary antibody, are illustrated in A and B. In C, swollen spores and germings exhibit constitutive acid trehalase activity originally present in ungerminated spores, close to the cell surface. In D, the acid trehalase signal was much stronger, consistent with the enzyme induction. Again, the enzyme was tightly packed covering the cell surface, and also appeared to accumulate at the hyphal tip (arrows).


