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Solubilization and characterization of a cell wall-bound trehalase from ascospores of the fission yeast *Schizosaccharomyces pombe*

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Summary

The genome of the fission yeast *Schizosaccharomyces pombe* lacks sequence homologs to *ath1* genes coding for acid trehalases in other yeasts or filamentous fungi. However, acid trehalase activity is present at the spore stage in the life cycle of the fission yeast. The enzyme responsible for this activity behaves as a surface enzyme covalently linked to the spore cell walls in both wild-type and *ntp1* mutant strains devoid of neutral trehalase. Lytic treatment of particulated cell wall fractions allowed the solubilization of the enzyme into an active form. We have characterized this soluble enzyme and found that its kinetic parameters, optimum pH and temperature, thermal denaturation and salt responses are closely similar to other conventional acid trehalases. Hence, this rather unusual enzyme can be recognized as acid trehalase by its biochemical properties although it does not share genetic homology with other known acid trehalases. The potential role of such acid trehalase in the mobilization of trehalose is discussed.

Introduction

Trehalose plays a key physiological role in yeast cells as reserve and stress metabolite (van Laere, 1989). Enzymes that breakdown this storage disaccharide are specific hydrolases known as trehalases. Yeast trehalases are classified in two classes, neutral (regulatory) and acid (non regulatory) trehalases, on the bases of their optimum pH for activity, ability to become activated by covalent modification and localization into the cells (Thevelein, 1984). Neutral trehalases coexist with trehalose within the cytosol as cryptic enzymes whose activity can be triggered by cAMP-dependent phosphorylation of the enzyme protein. Acid trehalases are compartmentalized into vacuoles or at the cell surface and their activity is not regulated by post-traslational

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mechanisms (Thevelein, 1984). Recently, it has been suggested to rename neutral and acid trehalases as cytosolic and extracellular trehalases, respectively, to describe more adequately their localization and function in the yeast cells (Parrou et al., 2005). Trehalases with mixed biochemical properties have been described in thermophilic fungi (Lucio-Eterovic et al., 2005).

The implication of neutral trehalases in the hydrolysis of endogenous trehalose is well documented (Cansado et al., 1998). Besides, it is generally accepted that the main function of acid trehalases is related to the utilization of exogenous trehalose as carbon source (d'Enfert and Fontaine, 1997; Parrou et al., 2005). However, the involvement of acid trehalases in the catabolism of intracellular trehalose has been suggested in several reports. In particular, Inoue and Shimoda (1981) proposed that the mobilization of endogenous trehalose in the fission yeast *Schizosaccharomyces pombe* during spore germination might be carried out by a cell wall-bound acid trehalase. They also suggested that the germination stimuli would alter plasma membrane permeability to allow the cytosolic trehalose to reach the trehalase enzyme located outside the spore. Later work demonstrated that in addition to the sporulation-specific acid trehalase, a cytosolic neutral trehalase exists in the vegetative cells of the fission yeast, leading to the assumption that such enzyme would be responsible for the mobilization in spores of the stored trehalose (De Virgilio et al., 1991). However, subsequent studies described that trehalose was still mobilized during germination in spores from *Sch. pombe* mutants defective in neutral trehalase, although at a much lower rate than in wild type spores (Beltran et al., 2000). Moreover, inhibition of the acid trehalase in these mutants blocked trehalose catabolism and greatly retarded spore germination, suggesting an important role in the breakdown of this reserve carbohydrate during the germination process (Beltran et al., 2000). The decrease in trehalose content observed in the absence of neutral enzyme was attributed to the acid trehalase, which was thus considered to participate in the hydrolysis of the endogenous trehalose as an ancillary enzyme. In contrast to all biochemical data supporting the presence of an acid trehalase activity during sporulation, the complete sequencing of the *Sch. pombe* genome revealed the absence of sequence homologs to known acid trehalases from other yeasts and fungi (Parrou et al., 2005). This unexpected finding has hampered direct genetic approaches in studies on the nature and physiological significance of the acid trehalase of *Sch. pombe*. In view of these conflicting results, we reinvestigated the trehalase enzyme of the fission yeast spores to present additional data related to its localization, solubilization and biochemical characterization.

Materials and methods

Cell strains, culture and ascospore isolation

Sch. pombe strain 968 (h^{90} *ura4-D18*) and strain MMT48 (h^{90} *ura4-D18 ntp1:: ura4*$^+$), which lacks neutral trehalase activity, were used in this study (Beltran et al., 2000). To obtain spores, cells were pre-grown until mid-exponential phase in YES liquid medium containing 2% glucose plus 0.6% yeast extract, washed in sterile distilled water and further incubated for 6 days in liquid MEL sporulation medium, which contained 3% malt extract in 50 mM sodium phosphate buffer pH 5.9 (Gutz et al., 1974). For ascospore isolation, samples of sporulated cultures were subjected to isopycnic centrifugation on linear density gradients of urografin (25–55%, v/v) in a swinging-bucket rotor at 25,000*g* for 30 min and 4 °C (Nishi et al., 1978). The samples resulted partitioned after centrifugation in three distinct isolated bands that were analyzed by phase-contrast microscopy. The upper fraction contained cellular debris and unbroken asci while the intermediate fraction was composed of remaining whole vegetative cells. Isolated intact ascospores were located purified in the band of more density (45–50% urografin), extensively washed with distilled water at low-speed centrifugation, resuspended in assay buffer and directly used as enzyme source in most experiments.

Treatment of spores, preparation of cells extracts and cell walls

Mild acid treatments of spores were performed essentially as indicated by Arnold (1972). Briefly, spores were subjected to 1-h treatments at room temperature in 0.1 N HCl or 3 N H_3PO_4 and then exhaustively washed on the centrifuge with 50 mM acetate buffer, pH 4.6. The percentage of live cells was ascertained by the methylene blue test in conjunction with a hemocytometer (Arnold, 1972). In parallel, spore viability was determined by the plate forming units method. Cell-free extracts from treated spores were prepared by mechanical disruption with glass beads as described previously (Carrillo et al., 1992) and tested for trehalase and marker enzymes against cell extracts from

untreated spores. Isolated cell walls were obtained according to Pérez and Ribas (2004). The lysing enzyme complex from *Trichoderma harzianum* (Glucanex; Novozyme Corporation, Sigma), which was found free of trehalase activity, was used to treat spore cell walls. The cell walls were washed in 50 mM acetate buffer pH 4.6, resuspended to an OD_{600} of 1.0 in the same buffer containing 20 μl/ml Glucanex and the suspension was incubated at 30 °C with shaking. The resulting supernatants were monitored every hour for trehalase after elimination of the glucose background by gel filtration as indicated below.

Enzyme assays

In trehalase assays, 0.25 ml of either spore suspension (10^9 spores/ml) or the corresponding cell extract was added to 0.25 ml containing 200 moles trehalose in 50 mM acetate buffer pH 4.6. Sodium azide (1 mM) was routinely included in these assays to reduce potential uptake of the resulting glucose. After incubation at 30 °C with shaking the reactions were stopped by heating. The assay mixtures were centrifuged at low-speed centrifugation and the glucose originated in the reactions was measured in the supernatants by the glucose oxidase–peroxidase method (Carrillo et al., 1994). One enzyme unit released 1 nmol of glucose per min under the stated conditions. The intracellular enzymes alkaline phosphatase and α-glucosidase were used as reference markers in experiments for enzyme location and their activity determined according to Fernández et al. (1995). Maltase activity was measured as for α-glucosidase but with maltose as substrate. Assays for trehalase enzyme activation by phosphorylation were performed as described previously (Arguelles et al., 1986).

Gel filtration and non-denaturing electrophoresis

Elimination of glucose from lysate samples was performed by gel filtration using small Sephadex G-50 packed columns and recovering the enzyme in the elution void volume. The relative molecular weight of trehalase solubilized from the cell walls was estimated by gel filtration through a Sephadex G-200 column (60 cm × 2.5 cm) equilibrated with assay buffer. The elution pattern was calibrated using the following markers: dextran blue (2000 kDa), ferritin (450 kDa), catalase (240 kDa) and aldolase (158 kDa). The trehalase was resolved under native conditions by electrophoresis through 20 cm long, 5% polyacrylamide gels. The gels were run for 24 h at 250 V and trehalase activity was detected *in situ* by staining as described by Moreno et al. (1990) except that sucrose was changed for trehalose.

Results

Trehalase activity in ascospores

Intact ascospores of the wild type *Sch. pombe* strain 968 showed enzyme activity in standard acid trehalase assays. Table 1 shows a typical result on the distribution of the enzyme activity among the different bands obtained after fractionation of sporulated cultures by isopycnic centrifugation (see Materials and Methods). When cell samples of the resulting bands were washed and used as enzyme source without previous breakage, trehalase activity correlated exclusively to the presence of the undisrupted ascospores. No trehalase was detected associated to intact vegetative cells.

Since *Sch. pombe* cells of the homothallic strain 968 also contain a neutral trehalase it could be argued that the potential contribution of such enzyme to the trehalose breakdown was not completely dismissed in the above assays. To avoid miss-interpretation of the results we used in subsequent experiments ascospores from the *ntp1*-disrupted strain MMT48, which is isogenic to strain 968 but devoid of neutral trehalase activity due to disruption of the *ntp1* gene (Beltran et al., 2000). The results showed that whole ascospores of *Sch. pombe* lacking neutral trehalase also hydrolyzed trehalose, pointing to the existence of an independent enzyme able to cleave this disaccharide.

Localization of acid trehalase

Exposure of intact ascospores from the MMT48 strain to mild acid treatments completely inactivated

Table 1. Distribution of trehalase activity after isopicnic centrifugation of cell cultures in linear gradients of urografin (25–55%)

Fraction	Trehalase (units/assay)	Specific activity (units/10^9 cells)	Content
Upper	0	0	Cell debris
Intermediate	0	0	Vegetative cells
Bottom	2.7	28.4	Spores

the acid trehalase activity originally present in untreated samples under conditions in which more than 90% of the spores remained viable. In contrast, as summarized in Table 2, the acid treatments did not significantly affect the activity of intracellular enzyme markers. These results are congruent with the interpretation that the trehalase enzyme is located at the cell surface. The fact that acid-treated *ntp1*-disrupted spores contain almost a full complement of intracellular marker enzymes while they are no longer potential sources for trehalase activity, together with the observation of a rapid inactivation of the solubilized enzyme at pH values below 2.0 (see later), supports that this trehalase must be located external to the cell membrane.

To gain more insight into the localization of the enzyme we devised an assay method for trehalase determination alternative to the original glucose-oxidase/peroxidase procedure. The assay was based on the use of eugenol (2-metoxi-4-alil-fenol) instead of the chromogenic oxidized substrate *o*-dianisidine. The complete procedure involved a coupled reaction between the oxidation of the glucose generated from trehalose by trehalase and the oxidation of eugenol by peroxidase, which results in polymerized eugenol as a white insoluble precipitate where glucose is being released. The rationale behind this approach was that if glucose was being produced outside the cells, the formation of a white precipitate in the enzyme assays would then be observed. The data supported that glucose originates extracellularly in assays with intact ascospores and exogenously added trehalose. These findings suggested that the enzyme was either periplasmic or cell wall-located *in vivo*. Additional evidence for the external location of this trehalase derived from fractionation of spore cell extracts. After low speed centrifugation (3000*g* for 5 min), the bulk of the enzyme activity present in the extracts (>90%) was in the particulated fraction and recoverable in the cell wall-rich pellet obtained after exhaustive washings.

Fractions enriched in cell walls were also assayed for maltase and α-glucosidase and no activity was found. The absence of activity in these fractions containing acid trehalase using *p*-nitrophenyl-α-glucoside as substrate is congruent with the observation that trehalase is acid-sensitive *in vivo* whereas α-glucosidase is not (Table 2).

Table 2. Relative location of acid trehalase in ascospores from *ntp1*-disrupted *Sch. pombe*

	Treatment of spores		
	None	0.1 N HCl for 10 min	3 N H_3PO_4 for 10 min
Viability (methylene blue test)[a]	99.1%	96.2%	88.7%
Viability (plate counts)[b]	100%	83.2%	76.4%
Alkaline phosphatase[c]	515.1	502.6	461.4
α-glucosidase[c]	283.4	266.1	230.7
Acid trehalase[d]	27.9	<0.1	<0.1

[a]Percentage of live cells as judged by methylene blue test.
[b]Colony formation ability after 6-day incubation.
[c]Enzyme activities are given as units per 10^9 cells determined in spore extracts.
[d]Enzyme activity given as units per 10^9 cells determined in whole spores.

Solubilization of the cell wall-bound trehalase

Attempts to release the acid trehalase by treating the particulated fractions with solutions of increased NaCl concentrations or 2% SDS failed to render soluble the acid trehalase activity in the corresponding supernatants, suggesting a covalent linkage (Cassone et al., 1979; Pérez and Ribas, 2004). The cell wall fractions were then treated at 30 °C with the lytic complex Glucanex, which is able to partially degrade the ascospore envelope. The resulting enzyme lysates showed a high glucose content, which seriously hindered trehalase determinations, likely arising from partial hydrolysis of the cell wall structural glucans. Hence, to perform in the absence of glucose a follow-up of the potential cell wall-bound trehalase being released, the supernatants of the lysates were analyzed for trehalase determination after passing through small Sephadex G-50 columns and employing the obtained void volume fractions as enzyme source. Fig. 1 shows data on the decrease of trehalase in the particulate fraction and its concomitant appearance as soluble form upon treatment with the lysing complex. Typical results indicated that about 60% of the original enzyme activity was recovered as free active form after 4 h of incubation with Glucanex (Fig. 1).

Properties of the solubilized enzyme

The trehalase solubilized from the ascospore cell walls showed a broad elution pattern by gel filtration chromatography in Sephadex G-200 (Fig. 2). Moreover, when the activity was detected on polyacrylamide gels by several *in situ* staining procedures after non-denaturing electrophoresis (Moreno et al., 1990; Sánchez et al., 2003) a fuzzy

migration was observed (not shown). These results were consistent with an enzyme of glycoprotein nature, as expected from a surface location. In addition, they suggested various degrees of glycosylation in the enzyme protein, likely arising from alterations in the original carbohydrate moiety by the hydrolytic activities present the enzyme complex employed for its solubilization. This interpretation would also explain the unusual large MW shown by gel filtration, between 250 and 400 kDa. Both the free and the cell wall-bound enzyme showed a K_m for trehalose of 10.9×10^{-3} mM and optimum pH for activity at 4.6 (Fig. 3). These values are within the range of typical acid trehalases in yeasts (Thevelein, 1984; Parrou et al., 2005).

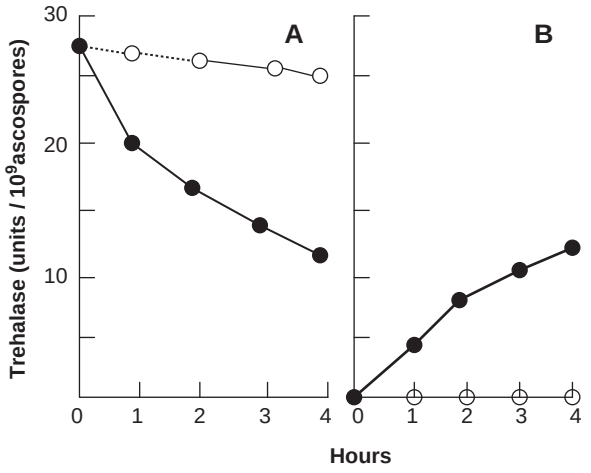

Figure 1. Enzymic treatment of the cell wall fraction from spore extracts. Samples were suspended in assay buffer and incubated at 30 °C in the presence (●) or absence (○) of 4 mg/ml Glucanex and 1 mM PMSF. At time intervals aliquots were removed and centrifugued at 10,000*g* for 2 min. The supernatants obtained were saved and the particulate fractions extensively washed with assay buffer. Trehalase activity was measured in both the particulate (residual activity, panel A) and the supernatant fractions (soluble activity, panel B) and expressed as enzyme units per 10^9 original spores.

A distinct feature of acid trehalases from yeasts, as opposed to neutral trehalases, is their inability to become activated by phosphorylation. Hence, assays were performed containing cAMP, ATP, protein kinase and the solubilized trehalase under conditions in which neutral trehalases from *Saccharomyces cerevisiae* and other yeasts are activated (Arguelles et al., 1986). In agreement with the genetic disruption present in the strain utilized, no activation was detected, indicating that the enzyme measured was not a neutral trehalase. Moreover, ATP alone did not inhibit the enzyme activity (Table 3). In contrast, neutral trehalases are known to be inhibited by ATP (Lucio-Eterovic et al., 2005)

The trehalase enzyme showed a relatively high thermal stability, being practically unaffected by 10 min treatments at 50 °C. The temperature optimum under the assay conditions was 60 °C (Fig. 3), which is higher than the values reported for conventional neutral trehalases (Thevelein, 1984). Moreover, the enzyme was not activated by Ca^{2+} ions and was resistant to EDTA inhibition (Table 3), which are typical properties of acid trehalases (Londesborough and Varimo, 1984; Lucio-Eterovic et al., 2005)

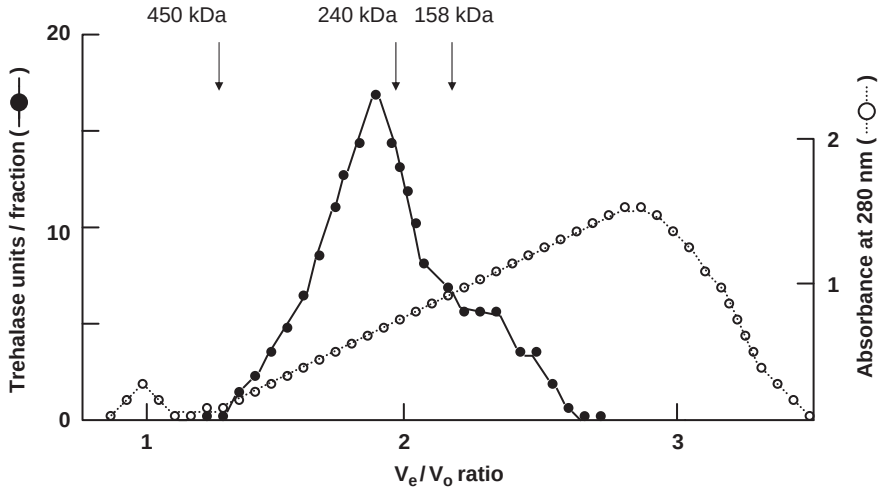

Figure 2. Elution pattern in Sephadex G-200 gel filtration of the acid trehalase solubilized from spore cell walls after a 4-h treatment with Glucanex. The arrows show the elution peak of protein markers of known molecular weights (ferritin, 450 kDa; catalase, 240 kDa; aldolase, 158 kDa). V_e, elution volume, V_o, void volume as determined by blue dextran 2000.

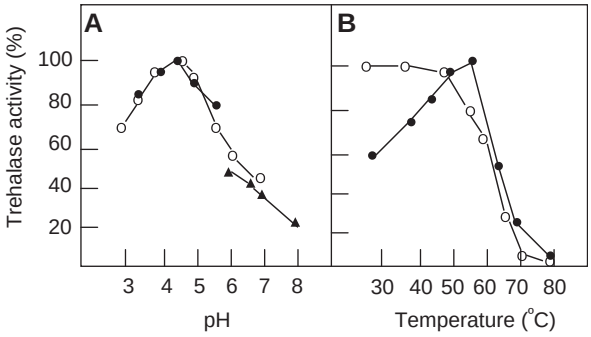

Figure 3. (A) pH profile of the trehalase activity. Enzyme samples were solubilized from the cell wall and dialyzed overnight against distilled water in the cold. Trehalase assays were performed under standard conditions of substrate concentration, temperature and incubation time except that different pH values were used. Buffers employed were citrate-phosphate (3.0–7.0; open circles), acetate (3.6–5.6; closed circles) and phosphate (6.0–7.8; triangles) at the indicated pH ranges. (B) Temperature optimum and thermal inactivation of the trehalase activity. Assays were performed with dialyzed enzyme under standard conditions except that different temperatures were used for incubation of the mixtures (closed symbols). In parallel, enzymes samples were incubated at different temperatures in the absence of substrate. At the indicated times samples were withdrawn, chilled and assayed for residual trehalase activity (open symbols).

Table 3. Effect of ATP and calcium salts on the solubilized enzyme

Assay conditions	Trehalase activity (units/mg protein)
Control	18.3±0.9
+ATP (5 mM)	18.0±0.7
+ATP (10 mM)	17.7±0.9
+Ca^{2+} ($CaCl_2$, 1 mM)	18.1±0.3
+Ca^{2+} ($CaCl_2$, 5 mM)	17.9±1.1
+EDTA (10 mM)	18.4±0.2

Fractions from the peak of trehalase activity after Sephadex G-200 gel filtration were used. Results are the mean value±SD from three independent determinations.

Discussion

The notion that trehalase is present in the cell wall of ascospores was already hypothesized by Hecker and Sussman (1973). By using new approaches, we confirm the existence in spores of *Sch. pombe* of a surface-bound enzyme able to break exogenous trehalose and present evidence for covalent linkage of this enzyme to structural components of the ascospore wall. When released from the cell wall architecture, the soluble enzyme maintains catalytic activity on trehalose and shows kinetic parameters that are indicative of a trehalase pertaining to the so-called acid or extracellular type (Thevelein, 1984; Parrou et al., 2005). Apparently, this enzyme is expressed in *Sch. pombe* only during sporulation (De Virgilio et al., 1991; and our own results).

Acid accessibility has been exploited to locate various enzymes at the surface of fungal spores (Mandels, 1953). Cells can be subjected to controlled mild acid treatments that destroy most of their periplasmic enzyme activity without killing the cells nor interfering seriously with other properties (Arnold, 1972). The results may supply a preliminary indication of enzyme localization outside the cell membrane. We have used this approach to locate acid trehalase in *ntp1*-disrupted ascospores, which lack neutral trehalase, and the initial evidence has been confirmed by a variety of different tests. The external distribution of this enzyme in spores of the fission yeast appears similar to that of acid trehalase in vegetative cells of *Candida albicans*, which is a structural component of the cells wall (Pedreño et al., 2004), but it is rather different from that of the extracellular acid trehalase in *S. cerevisiae*, which is mainly found soluble within the periplasmic space (Jules et al., 2004).

Acid trehalases from *S. cerevisiae*, *C. albicans*, *Aspergillus nidulans* and other species share several common features, like the presence of the glycosyl hydrolase N-terminal and the central catalytic Pfam domains that characterize their classification in the GH65 CAZy family (Parrou et al., 2005). Surprisingly, the genome of the fission yeast *Sch. pombe* does not contain any recognizable ORF homolog to other acid trehalases from yeasts or filamentous fungi. The situation is even more intriguing because the genome of *Sch. pombe* only contains a single *ntp1* gene coding for neutral trehalase and, contrariwise to *S. cerevisiae*, no analogs are found with similar function (Cansado et al., 1998). Notably, *S. cerevisiae* possesses two genes which encode two protein isoforms of neutral trehalase with high sequence identity (Coutinho et al., 2003). Deletion of one gene is associated with a lack of measurable *in vitro* trehalase activity (Nwaka and Holzer, 1998) but degradation of trehalose *in vivo* may occur due to the expression of the other gene (Parrou et al., 2005). By contrast, in mutant strains of *Sch. pombe* lacking neutral trehalase no obvious alternative enzyme activity exists to explain the breakdown of trehalose other than acid trehalase.

Earlier observations on trehalose mobilization in spores of *Sch. pombe* suggested the existence of a

mechanism during germination that would require the export of the disaccharide, its extracellular hydrolysis, and the subsequent uptake of the glucose released (Inoue and Shimoda, 1981; Beltran et al., 2000). This hypothesis has been in part supported by the observation that the deletion of *ath1*$^{+}$ (encoding acid trehalase) in *nth1*$^{-}$*nth2*$^{-}$ double mutant cells of *S. cerevisiae* (which lack the two genes coding for neutral trehalases) results in the accumulation of extracellular trehalose (Parrou et al., 2005). Data presented here concerning the enzyme location are thus compatible with the above interpretation.

Several lines of evidence support that in spite of lacking common sequences, the cell wall-located enzyme of *Sch. pombe* shares properties similar to other acid trehalases from different yeasts species. First, not only its optimum pH is within the acid range, but it exhibits both a temperature optimum and a thermal resistance higher than neutral trehalases (Thevelein, 1984). Second, it is insensitive to calcium salts and lacks inhibition by nucleotides such as ATP. Finally, like many other acid trehalases, it is located outside the cell and, hence, far from the post-translational control which regulate the activity of neutral trehalases. However, it does not appear to be inhibited by acetate, unlike other reported acid trehalases (Fig. 3) (Londesborough and Varimo, 1984; Arguelles and Gacto, 1985).

Taken as a whole, the results presented in this study add insight into the nature and physiological significance of the acid trehalase in the fission yeast, which was biochemically recognized as such by several workers before fully sequencing of the fission yeast genome. The solubilization of this enzyme favors its purification and may eventually facilitate the design of molecular probes for the identification of the corresponding gene within the *Sch. pombe* genome. The enzyme might represent a novel class of trehalases not fitting among the types so far established on the bases of sequence homology. Further characterization of enzymes similar to this particular hydrolase of the spore coat of *Sch. pombe* may help to broaden the knowledge on trehalases and their role in yeast biology.

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