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A constitutive, heat shock-activated neutral trehalase occurs in *Schizosaccharomyces pombe* in addition to the sporulation-specific acid trehalase

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1. SUMMARY

Trehalase was studied in *Schizosaccharomyces pombe* cells growing vegetatively on minimal medium and in sporulating cultures. Acid trehalase activity, measured at pH 4.2, was absent in vegetative cells and occurred only in asci, indicating that this activity represented the sporulation-specific trehalase reported previously. In contrast, neutral trehalase, measured at pH 6.0, was constitutively present in vegetative cells during the exponential and stationary growth phase as well as in asci. In vegetative cells, neutral trehalase did not sediment with cell walls, suggesting a cytoplasmic localization. Its activity increased ten-fold when growing cells were subjected to heat treatment of 2 h. Neutral trehalase from heat-treated cells had a pH optimum of 6.0 and was almost completely inhibited by 3 mM $ZnCl_2$. Acid trehalase activity could be measured in intact asci, indicating that it is localized in the ascus cell walls, while neutral trehalase was not detectable in intact asci and appeared to be present primarily in the walls of ascospores and in the ascus epiplasm.

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2. INTRODUCTION

Trehalose, a non-reducing disaccharide (α-D-glucopyranosyl 1,1-α-D-glucopyranoside) is distributed ubiquitously in fungi and is thought to play a role as storage carbohydrate [1] and/or as protectant against environmental injuries [2,3]. In *Schizosaccharomyces pombe* [4,5], as in *Saccharomyces cerevisiae* and many other fungi (for a review see [1]), trehalose accumulates preferentially during sporulation immediately before the spores reach maturity; conversely, the accumulated trehalose is rapidly mobilized again at the onset of spore germination and thereafter is barely detectable in the growing vegetative cells.

In fungi, trehalose degradation has invariably been found to proceed via hydrolysis catalysed by

trehalases. The fungal trehalases have been divided into two groups [1]: (i) the neutral trehalases with a pH optimum between 6.0 and 7.5, called regulatory trehalases because they can be activated by cAMP-dependent protein phosphorylation and (ii) the acid trehalases with a pH optimum between 3.5 and 5.5, called non-regulatory because there is no evidence for rapid regulation. Both types occur in *S. cerevisiae* and have been studied extensively: the neutral trehalase, a Zn^{2+}-sensitive enzyme, is located in the cytoplasm [6–9], while the acid trehalase, a Zn^{2+}-insensitive enzyme, is a vacuolar glycoprotein [7,10,11].

In contrast, it has been reported that *S. pombe* lacks trehalase in vegetative cells and possesses only a sporulation-specific cell wall-bound trehalase [4,5] which has been assigned to the non-regulatory type [1]. However, we found recently that *S. pombe* also possesses a trehalase activity in vegetative cells which strongly increases upon heat shock [12].

Here, we compare the trehalase activities present in vegetative cells and in asci of *S. pombe*, and we demonstrate that in addition to the sporulation-specific trehalase which is an acid, Zn^{2+}-insensitive enzyme located in the cell walls of asci, a constitutive neutral Zn^{2+}-sensitive trehalase occurs both in vegetative cells and in asci.

3. MATERIALS AND METHODS

3.1. Organisms and culture conditions

Schizosaccharomyces pombe wild type strains 972 h^- and 975 h^+ (received from P. Nurse, Oxford University, U.K.) were kept as stock cultures on YPDA (yeast extract 1%, bactopeptone 2%, glucose 2%, agar 2%) and grown at 27 °C on a shaker (140 rpm) in liquid Edinburgh Minimal Medium No. 2 (EMM2) [13].

Conjugation was performed by mixing two cultures in the exponential growth phase (10^7 cells/ml) of the strains 972 h^- and 975 h^+ pregrown on EMM2. The culture was allowed to grow to the stationary phase and was then transferred to sporulation medium (EMM2 without nitrogen source, half of the vitamins and only 1% glucose). Asci were harvested 48 h after the transfer of the diploids to the sporulation medium.

3.2. Homogenization and cell fractionation

Asci were collected by centrifugation at 1000 $\times g$ for 10 min, suspended in 10 mM 2-(*N*-morpholino) ethane sulfonic acid (MES) adjusted to pH 6.0 with KOH, and used in the trehalase assay, either intact or after disruption by shaking with glass beads [12]. Enzyme preparations of cells in the exponential and stationary growth phase were prepared after mechanical disruption by glass beads as previously described [12].

Fractionation of asci was performed as follows. The ascus walls were disrupted by sonicating with 180 W for 20 min, cooling after each min for 1 min in an ice bath. This treatment did not disrupt the ascospores. The suspension was centrifuged in conical centrifuge tubes at 20 000 $\times g$ and 4 °C for 10 min to separate the supernatant (soluble fraction, containing the ascus cytosol or epiplasm) from the sediment (containing ascus wall fragments and intact ascospores). The sediment was resuspended in 10 mM MES(K^+) buffer, pH 6.0, and vigorously shaken with glass beads [12] until at least 95% of the ascospores were disrupted as checked by microscopy. The homogenate was then centrifuged again at 20 000 $\times g$ and 4 °C for 10 min. The resulting supernatant fluid contained the ascospore cytosol, whereas the sediment contained ascus and ascospore wall fragments.

3.3. Determinations of trehalase activity and protein

Enzyme preparations were incubated in 100 mM trehalose, 0.2 mM $MnCl_2$ and either 80 mM citrate(K^+) buffer, pH 4.2, or 80 mM MES(K^+) buffer (pH 6.0) for 10–30 min. In order to avoid sedimentation of asci, ascospores and cell wall debris, the mixtures were shaken on a reciprocal shaker at 60 rpm during the assay. The reaction was stopped in a boiling water bath (3 min). The mixture was clarified by centrifugation and used for determination of glucose using the GOD test kit from Boehringer (Mannheim, F.R.G.) [12]. To determine the pH dependence, 80 mM buffers were prepared with acetic acid, citric acid, MES,

piperazine-*N*,*N*'-*bis*(2-ethane sulfonic acid) (PIPES) or *N*-Tris(hydroxymethyl)-methylglycine (TRICINE), adjusted with KOH.

Protein was determined as before [14] using bovine serum albumin as a standard.

4. RESULTS AND DISCUSSION

We found recently that growing vegetative cells of *S. pombe* contain a neutral trehalase [12], a result in apparent contradiction to previous work of Inoue and Shimoda [4,5] who measured trehalase at pH 5.6 and found activity only during ascus formation in sporulating cells. To investigate this discrepancy further, we studied trehalase activities at pH 4.2 and pH 6.0, conditions that would reveal specifically the acid and neutral trehalase in the case of *S. cerevisiae* [9,11]. Total homogenates of vegetative cells of *S. pombe* were devoid of any detectable acid trehalase activity at pH 4.2, both during exponential growth and in the stationary phase, but had a constitutive neutral trehalase activity at pH 6.0 (Table 1).

In contrast, asci had about the same trehalase activity at pH 4.2 and pH 6.0 (Table 1). This indicated that acid trehalase was the sporulation-specific activity reported previously while neutral trehalase was constitutive during the life cycle of *S. pombe*.

When the homogenates of growing or stationary vegetative cells were centrifuged at $1000 \times g$ for 10 min to sediment the cell walls, most of the activity (> 80%) of neutral trehalase remained in the supernatant. Thus, neutral trehalase in vegetative cells is not associated with cell walls but is probably a soluble cytoplasmic enzyme, as are all the well-characterized neutral trehalases of fungi [1].

Table 1

Specific activities of acid and neutral trehalases in *S. pombe*

	Specific activity (μkat/g protein) [a]	
	Acid trehalase (pH 4.2)	Neutral trehalase (pH 6.0)
Vegetative cells		
During exponential growth	< 0.01	0.12
In stationary phase	< 0.01	0.13
After heat shock [b]	< 0.01	1.24
Asci	0.16	0.19

[a] Specific activities were measured in total homogenates after cell disruption with glass beads.

[b] Exponentially growing vegetative cells were incubated at 40 °C for 2 h.

We examined the effect of a heat shock (2 h at 40 °C) on trehalase activity in growing vegetative cells. As reported previously [12], neutral trehalase activity increased about ten-fold under these conditions; acid trehalase remained below the detection limit (Table 1). Since induction of neutral trehalase occurs even in the presence of cycloheximide [12], it appears that the activity is regulated post-translationally, as in the case of the neutral trehalases of other fungi [1].

The neutral trehalase of *S. cerevisiae* can be activated in vitro by phosphorylation with cAMP-dependent protein kinase [6,8,9] and inactivated in vitro by alkaline phosphatase [9]. We incubated extracts from *S. pombe* under the appropriate conditions for activation and inactivation of *S. cerevisiae* neutral trehalase [9] but found no changes of trehalase activity (data not shown). Thus, the mechanism of the apparent post-translational regulation of neutral trehalase of *S. pombe* remains to be elucidated.

However, the neutral trehalase of *S. pombe* shares another property with that of *S. cerevisiae*: it is sensitive to Zn^{2+}, being 80% inactivated by 1 mM and almost completely by 3 mM Zn^{2+} (Fig. 1).

The pH dependence of trehalase activity was determined in different preparations (Fig. 2). In an extract from heat-shocked cells, a sharp optimum was observed at pH 6.0 (Fig. 2A), a value typical for neutral trehalases [1]. In a total homogenate of mature asci disrupted by shaking with glass beads, a treatment which also efficiently disrupted the ascospores, the pH profile suggested the existence of two different trehalases, a neutral trehalose with a pH optimum around pH 6 and an acid trehalose with a pH optimum at 4.2 (Fig. 2B). When the assays were performed in the presence of 12 mM $ZnCl_2$, the optimum at pH 6 completely disappeared, revealing the pH profile of a typical acid trehalase

[1,11] with an optimum at pH 4.2 (Fig. 2B). This finding shows that asci contain a Zn^{2+}-sensitive neutral trehalase similar to the vegetative cells and, in addition, a Zn^{2+}-insensitive acid trehalase. A pH profile typical of acid trehalase was also found when intact asci were used in the trehalase assay (Fig. 2C). Thus, the acid, sporulation-specific trehalase, but not the neutral trehalase, was readily accessible from the incubation medium, suggesting that the former was localized outside the ascus plasmalemma and the latter inside.

To study trehalase localization further, asci were fractionated as described in MATERIALS AND METHODS. Acid trehalase was measured at pH 4.2. Most of this activity could be measured already by incubating intact asci, namely 85% of the total activity present in ascus homogenates (Table 2). Upon further fractionation, this activity pelleted with cell wall fractions (Table 2: Sediment 1 and 2). The activity also sedimented upon low-speed centrifugation ($1000 \times g$ for 10 min). These results indicate a localization in the ascus walls. The loss of about 30% during fractionation can be explained by the cell disruption method used, where ascus wall fragments were partially lost, because they remained between the glass beads. Thus, the sporulation-specific acid trehalase is associated with the ascus cell wall. It

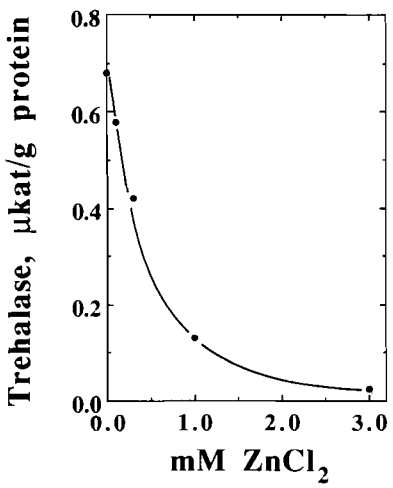

Fig. 1. Inhibition of the activity of the soluble neutral trehalase of log-phase cells by $ZnCl_2$.

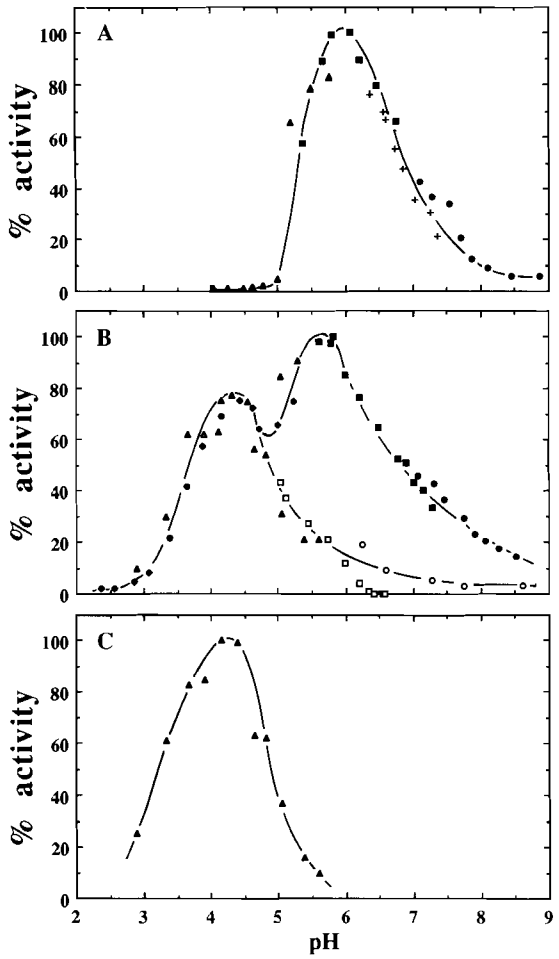

Fig. 2. pH-dependence of trehalase activity in various enzyme preparations. **A.** Soluble fraction of log-phase cells subjected to a 2-h heat shock at 40 °C for activation of trehalase. **B.** Total homogenate of asci with (open symbols) and without (closed symbols) 12 mM $ZnCl_2$. **C.** Intact asci. Assay buffers were either 80 mM acetic acid (triangles), citric acid (diamond), MES (squares), PIPES (cross) or TRICINE (circles) adjusted with KOH.

appears to be strongly bound, since it could not be released from the cell wall fractions by incubation in the presence of 0.5 M KCl for 20 min at 20 °C.

In contrast to the acid trehalase, most of the neutral trehalase (79%) was measured only after disintegration of the ascus walls (Table 2). A large part (approx. 70%) of this activity was sedimentable before, as well as after, disruption of

Table 2

Distribution of acid and neutral trehalases in fractions obtained from asci of *S. pombe*

	Activity			
	Acid trehalase (pH 4.2)		Neutral trehalase (pH 6.0)	
	Total (% [a])	Specific (nkat/g pr.)	Total (%)	Specific (nkat/g pr.)
Total homogenate of asci (disrupted by glass beads)	100	164	100	195
Intact asci	85	nd [b]	21	nd
Sediment 1 after sonication (ascospores, cell walls of asci)	94	162	63	128
Supernatant 1 after sonication (ascus epiplasm)	3	91	19	686
Sediment 2 after homogenization (cell walls of asci and ascospores)	64	500	77	715
Supernatant 2 after homogenization (cytoplasm of ascospores)	3	16	4	25

[a] Activities in various fractions were calculated per 10^7 asci and expressed as a percentage of the activity in the total homogenate of 10^7 asci.

[b] Not determined.

the ascospores (Table 2: Sediment 1 and 2, respectively), indicating an association with the ascospore walls. A smaller part (19%) remained in supernatant 1, the fraction containing the cytoplasm (epiplasm) of the asci. Supernatant 2, the fraction containing the cytoplasm of the ascospores, had very little trehalase activity (4%). Since the epiplasm of asci derives morphogenetically from the cytosol of vegetative cells [15] it seems likely that the neutral trehalase of *S. pombe* is probably a cytoplasmic enzyme, both in vegetative and generative cells, as are the neutral trehalases of *S. cerevisiae* and other fungi [1]. The activity bound to the ascospore wall might have been adsorbed or even entrapped from the ascus epiplasm during ascospore development. Alternatively, it might represent a different, exoplasmic enzyme deposited in the ascospore cell wall through the forespore membrane [15], an ER-derived membrane that differentiates into the ascospore plasmalemma.

The existence of a neutral, potentially regulatory trehalase in *S. pombe* has new implications for the theory of compartmentation of trehalose metabolism during ascospore germination. It has been suggested that the trehalase associated with ascospore walls is involved in the hydrolysis of the trehalose stored in the cytoplasm ([4,5]; reviewed in [1]). According to this theory, trehalase is released from the spores to be hydrolysed in the extracellular space, a potentially wasteful process. It now seems possible that a regulatory neutral trehalase exists in a cryptic, inactive form in the cytoplasm of the resting ascospores and is activated upon germination, as is likely to occur in ascospores of *S. cerevisiae* and other fungi with a regulatory trehalase [1].

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