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Vacuolar (Lysosomal) Trehalase of *Saccharomyces cerevisiae*

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Abstract. In the yeast *Saccharomyces cerevisiae* the *PEP4* gene product, protease A, is responsible for activating all soluble vacuolar (lysosomal) enzymes. These vacuolar enzymes remain inactive in *pep4* mutants. Vacuolar trehalase activity was diminished in such mutants as well. This suggests that the vacuolar (lysosomal) trehalase is processed in a manner similar to other vacuolar enzymes in *S. cerevisiae*.

In the yeast *Saccharomyces cerevisiae*, the vacuole is functionally analogous to the eukaryotic lysosome [7, 10]. The vacuole contains a number of hydrolytic enzymes, primarily proteases, and also serves as a storage pool for amino acids, polyphosphate, and other compounds [4, 10].

Vacuolar enzymes are initially made as inactive proenzymes [4]. Vacuolar protease A cleaves the prosequence, thereby activating the enzymes. Protease A is encoded by the *PEP4* gene [1, 11]; *pep4* mutants possess diminished levels of all soluble vacuolar enzymes tested and accumulate the inactive proenzymes [4, 5].

Saccharomyces cerevisiae has two trehalases, one located in the vacuole and the other located in the cytoplasm [6]. We set out to determine whether the vacuolar trehalase is processed in a manner similar to other vacuolar enzymes by examining the effects of the *pep4* mutation on its activity.

Materials and Methods

Strains and media. The strains JHRY20-2C (a *his3-Δ200 leu2-3,112 ura3-52 PEP4*) and JHRY20-2Ca (a *his3-Δ200 leu2-3,112 pep4::URA3*) were obtained from Joel Rothman (Institute of Molecular Biology, University of Oregon, Eugene, Oregon).

Rich medium, YEPD, consisted of 1% Difco yeast extract (Difco, USA), 2% Bacto–peptone, and 2% glucose. Synthetic complete medium, SC, consisted of 0.67% Difco–yeast nitrogen base plus 2% glucose. Appropriate nutritional supplements were added at 30 mg/liter. For trehalose-based media, 2% trehalose (Sigma, USA) was substituted for glucose. For derepressing medium (YEP), 0.1% glucose was substituted for 2% glucose. For solid media, 2% agar (Oxoid, Canada) was added.

Growth of *pep4* mutants. Cultures were grown in 250-ml Erlenmeyer flasks (Bellco, USA) on a gyratory shaker (New Brunswick Scientific, USA) at 200 rpm.

Strains JHRY20-2C and JHRY20-2Ca were grown in either YEPD medium or YEP medium. Midexponential growth phase cells in YEPD medium were used to inoculate the cultures. Aliquots were removed for harvesting at the time of inoculation into fresh YEPD medium or YEP medium and following 8–12 h growth under derepressing or repressing conditions. Cell numbers in each culture were determined with the aid of a hemacytometer at the time of harvesting. The volume of each aliquot from separate cultures was adjusted such that there were equal cell numbers in each sample. The ability of these strains to grow on trehalose was monitored after 20 h in SC + Tre medium. Stationary-phase cultures from YEPD were harvested and washed. These were used to inoculate parallel cultures in SC and SC + Tre at an equal starting cell number.

Determination of trehalase activities. Cells were harvested by centrifugation at setting 6 in an IEC clinical tabletop centrifuge. The cells were washed once and were resuspended in 5 ml of sterile double-distilled water.

The cells were disrupted by three passages through a French Press chamber (Amicon, USA) at 20,000 psi. The French-pressed lysate was centrifuged at 12,000 rpm for 15 min in a Beckman JA-21 refrigerated centrifuge. The supernatant was used as a crude cell extract; however, the pellet was also examined for activity.

Trehalase activities in the crude cell extract were determined by the discontinuous trehalase assay with some modifications [3, 6]. Assays for vacuolar and cytoplasmic trehalase activities were conducted simultaneously. In the assay for vacuolar activity, 200 μl of crude extract was incubated with 400 μl of 50 m*M* MES buffer containing 1 m*M* EDTA at pH 5. Added to this was 200 μl of 12.5 m*M* trehalose suspended in the same buffer. In the assay for cytoplasmic activity, 200 μl of crude extract was incubated with 400 μl of 50 m*M* PIPES buffer containing 2.5 m*M* $CaCl_2$ at pH 7. Added to this was 200 μl of 12.5 m*M* trehalose suspended in this buffer. After a 1-h incubation at 23°C, the samples were incubated in a 90°C water bath for 10 min to terminate the reaction. It was initially determined that during this incubation the amount of trehalase present in the crude extract

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Table 1. Growth of a *pep4* mutant on trehalose-containing medium[a]

Strain	Media	Cell number ($\times 10^7$) 0 h	20 h
JHRY20-2C (*PEP4*)	SC	1.0	7.1
	SC + Tre	1.0	1.6
JHRY20-2Ca (*pep4*)	SC	1.0	6.8
	SC + Tre	1.0	1.4

[a] Stationary phase cells from YEPD were harvested. One-half of the cells were inoculated into SC, and the remaining half were inoculated into SC + Tre. Following 20 h of incubation, the cell number in each culture was determined with a hemocytometer. Representative results from one of five independent experiments are presented.

Table 2. Trehalase activities in a *pep4* deletion mutant[a]

Strain	Trehalase activity: Vacuolar Rep.	Vacuolar Drep.	Cytoplasmic Rep.	Cytoplasmic Drep.
JHRY20-2C (*PEP4*)	1.0	5.0	1.0	1.0
JHRT20-2Ca (*pep4*)	0.0	1.2	1.0	1.0

[a] Midexponential growth phase cells growing on YEPD were harvested. One-half of the cells were inoculated into YEPD (repressing conditions), and the remaining half were inoculated into YEP (derepressing conditions). The cultures were incubated for 8–12 h, after which they were harvested and crude extracts prepared. Representative results from one of ten independent experiments are presented. Specific activity is defined as the number of nmoles of glucose released per min per milligram of protein and are expressed as values normalized to the controls.

was rate limiting. Enzyme and substrate controls contained 200 μl of crude extract and 200 μl of 12.5 m*M* trehalose, respectively, suspended in 600 μl of buffer. For each assay, a series of glucose standards was included.

Liberated glucose was measured as described previously [3]. Protein was measured according to the Bradford method, with rabbit gamma-globulin as a standard [2].

Results

Growth of *pep4* mutants on trehalose. The ability of the *pep4* mutant JHRY20-2Ca and its parent strain JHRY20-2C to grow on trehalose-based media was compared. Each strain grew equally well on SC after 20 h incubation (Table 1). However, each strain exhibited similar poor growth on SC + Tre (Table 1). JHRY20-2C and JHRY20-2Ca each grew poorly on YEPT as well. The *pep4* mutant did not show a decreased ability to grow on trehalose compared with its parental strain.

Trehalase activities in *pep4* mutants. Trehalase activities in JHRY20-2C and JHRY20-2Ca were determined in cells subjected to derepressing conditions. They were examined on derepressing media compared with repressing media, since these were the conditions that were used to study the effects of the *pep4* mutation on other vacuolar enzymes [5]. This was also done to negate any possible effects that the exogenous trehalose might have had on the trehalase activities.

No vacuolar trehalase activity was present in JHRY20-2Ca when it was grown under repressing conditions. The parent strain JHRY20-2C had a low level of activity (Table 2). Under derepressing conditions, there was a fourfold reduction in vacuolar trehalase activity in the *pep4* mutant compared with its parent (Table 2). Each strain exhibited similar levels of cytoplasmic trehalase activity under repressing and derepressing conditions (Table 2).

Discussion

The *PEP4* gene product, protease A, is required to cleave the prosequence from vacuolar proenzymes once they arrive in the vacuole, thus activating them [1, 4, 11]. In the absence of this activity, all vacuolar enzymes tested to date show diminished activity. In order to determine whether the *PEP4* gene had an effect on the cytoplasmic or vacuolar trehalases, the activities of these enzymes were determined in a *pep4* mutant. JHRY20-2Ca carries a complete *pep4* deletion, generated by the insertion of the selectable URA3 gene into the *PEP4* coding region such that all residual activity is eliminated [1]. Trehalase activities were examined on derepressing medium compared with repressing medium, since these conditions were used to study the effects of the *pep4* mutation on the other vacuolar enzymes.

In JHRY20-2Ca, there was no trehalase activity under repressing conditions. Under derepressing conditions, about 20% of the wild-type level of activity was present. The cytoplasmic trehalase activity was unaffected. These results clearly demonstrate that the vacuolar trehalase is dependent on protease A for activation, whereas the cytoplasmic activity is not. The presence of some active vacuo-

lar trehalase in the *pep4* mutant under derepressing conditions may be due to partial nonspecific activation of some accumulated proenzyme in the crude extract or to the uncovering of a *pep4*-independent trehalase activity in the vacuole.

It has been previously proposed that the presence of a vacuolar trehalase activity could be accounted for by the translocation of the cytoplasmic trehalase into the vacuole for proteolytic degradation subsequent to its activation by phosphorylation [9]. However, it has been demonstrated that there are substantial differences between the two activities, particularly that the vacuolar activity is accounted for by a glycoprotein, whereas the cytoplasmic activity is not [6]. Our results show that the vacuolar trehalase is processed in a manner different from the cytoplasmic trehalase and that their activities are also under different controls. The evidence indicates that the vacuolar trehalase is processed through the same pathway as other soluble vacuolar enzymes in *S. cerevisiae* [4]. These results, in addition to other evidence that vacuolar trehalase travels through the early stages of the secretory pathway (Harris and Cotter, unpublished results), imply that there exists a compartmentalized trehalase in *S. cerevisiae* in addition to the cytoplasmic trehalase regulated by phosphorylation [8].

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