Purification and biochemical characterization of the *ATH1* gene product, vacuolar acid trehalase, from *Saccharomyces cerevisiae*

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Abstract **The disaccharide trehalose plays a critical role in yeast cell survival during conditions of environmental stress. The vacuole of the yeast *Saccharomyces cerevisiae* contains an enzyme, acid trehalase (ATH), that is capable of degrading trehalose. Recently, a gene required for ATH activity, *ATH1*, was cloned and sequenced [Destruelle et al., (1995) Yeast 11, 1015–1025]. The relationship between *ATH1* and ATH, however, was not determined. We have purified ATH and shown that it is the *ATH1* gene product; peptide sequences from the purified protein correspond to the deduced amino acid sequence of Ath1p. In addition, antiserum to Ath1p specifically recognizes purified ATH.**

Key words: Acid trehalase; *ATH1* gene; Trehalose; Vacuole; Yeast

1. Introduction

Of the many physiological processes associated with the yeast vacuole, its degradative capacity is the most thoroughly characterized [2–4]. The numerous hydrolytic enzymes localized to this organelle play a role in the breakdown and turnover of a wide range of metabolites. The vacuole also serves as a site for the recycling of subcellular organelles [5,6]. In either case, the proteins or membrane-bound compartments destined for degradation need to enter the vacuole lumen where they become accessible to the reduced pH and hydrolytic environment. Following hydrolysis, the macromolecular constituents are either stored within the vacuole or released into the cytosol to be used in metabolic reactions.

Due to their potential degradative capacity, many vacuolar hydrolases are synthesized as precursor proteins that require proteolytic maturation and/or an acidic pH for optimal activity [7]. In some cases, other aspects of the vacuolar milieu such as the salt concentration may enhance the hydrolytic capacity [8]. These factors along with the compartmentalization of these enzymes within the organelle allow the hydrolases to be separated from potential substrates; this sequestration essentially serves a regulatory role by limiting potential enzymatic reactions. Astonishingly little is known, however, regarding the mechanisms by which metabolites are delivered to the vacuole and even less is understood about the efflux processes.

Carbohydrate metabolism provides one example of an important physiological process that is poorly characterized with respect to the vacuole. α-mannosidase (AMS) is the only known general glycosidase localized to the vacuole. In addition to AMS, the vacuole contains at least one other glycosidase, the acid trehalase (ATH). Relatively little research has focused on the biosynthesis of this enzyme. ATH is reported to transit through the secretory pathway [9,10], and its activity is dependent on the activity of PrA [11]. ATH is of particular interest because its substrate, trehalose, plays an important role as a membrane protectant under stress conditions [12–15]. Again, there are more questions than answers concerning the function of ATH in cellular physiology. To further understand the possible role of this enzyme in trehalose metabolism, Mittenbühler and Holzer purified the enzyme [16] and attempted to study its biosynthesis. Even though they achieved an over 7000-fold purification of ATH activity, it appears that they copurified an additional protein. This highly glycosylated protein, gp37 [17], was most likely the protein characterized in an earlier study [9].

Recently, we undertook an alternative approach to characterize ATH by first identifying the structural gene. We took advantage of the observation that the overproduction of proteins that transit through the secretory pathway leads to their expression at the cell surface [18,19]. Relying on an assay to detect surface acid trehalase activity, we cloned a gene termed *ATH1* that is required for ATH activity [1]. Surprisingly, analysis of the deduced amino acid sequence of the *ATH1* gene failed to reveal homology to trehalases or the presence of a consensus signal sequence cleavage site [20]. These findings made it unclear whether the *ATH1* gene was the structural gene for ATH or whether it encoded a regulatory factor. To differentiate between these possibilities, and to gain further insight concerning the role of ATH in regulating the cellular levels of trehalose, we purified the acid trehalase. Peptide sequences from ATH match the deduced amino acid sequence of the *ATH1* gene. In addition, antiserum raised to a *TrpE-ATH1* hybrid protein recognizes the purified ATH protein. These results indicate that *ATH1* is the structural gene for ATH (see also [21]).

2. Materials and methods

2.1. Strains and media

The strains of *Saccharomyces cerevisiae* used in this study were SEY6210 (*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ902 lys-801 suc2Δ9*) [22]; MDY1 (SEY6210 *Δygp1::URA3*) [17]; MDY3 (SEY6210 *Δath1::URA3*) [1]. The acid trehalase-overproducing strain was prepared by transforming wild-type strain SEY6210 with the 2μ

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Abbreviations: ATH, acid trehalase; Endo H, endo-β-*N*-acetylglucosaminidase H; IEF, isoelectric focusing; NTH, neutral trehalase; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PrA, proteinase A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; YNB, yeast nitrogen base; YPD, 1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose

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overproducing plasmid pDAT1.9, which carries the *ATH1* gene [1]. This strain is referred to as SEY6210/pDAT1.9 throughout this paper.

Yeast cells were grown in YPD or synthetic minimal medium (0.067% yeast nitrogen base (YNB), 2% glucose, and auxotrophic amino acids and vitamins as needed).

2.2. Antibody generation and immunodetection

A *XhoI-SalI* restriction fragment encoding 42% of the *ATH1* open reading frame was isolated from plasmid pDAT1.9 and cloned in-frame into the vector pATH2 [23] at the unique *SalI* site to create a *TrpE-ATH1* gene fusion. Hybrid protein was overproduced in *E. coli* and partially purified using described techniques [23]. Preparative quantities of the proteins were solubilized and further purified using SDS-PAGE. Gel slices containing the band of interest were excised, homogenized and used for immunization of New Zealand white rabbits. Western blots and chemiluminescent detection of proteins were carried out essentially as described [24]. Antiserum to Ath1p was used at a dilution of 1:10 000. Goat anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Hercules, CA).

2.3. Assays

To assay for acid trehalase activity, yeast cells (132 000 A_{600} units) were grown in YPD or SMD, harvested by centrifugation and lysed with glass beads. A crude cell extract was prepared by centrifugation of lysed cells at $10\,000 \times g$ for 60 min and discarding the pellet fraction. Acid trehalase was assayed by incubating 20 µl of diluted (1:100) crude extract in a reaction mixture containing 40 mM sodium citrate, pH 4.5, 1 mM EDTA and 200 mM trehalose in a total volume of 250 µl in a microcentrifuge tube. After 30 min of incubation at 37°C, the reaction was stopped by adding 300 µl of 0.1 M KH_2PO_4, pH 10, and boiling the mixture for 3 min, followed by a high-speed centrifugation at $13\,000 \times g$ for 5 min. The glucose concentration was determined enzymatically as described previously [25]. One activity unit of acid trehalase is defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of trehalose/min at 37°C at pH 4.5.

Assays for the marker enzymes α-mannosidase, α-glucosidase and NADPH-cytochrome *c* reductase were performed as described previously [25]. Protein concentrations were determined using the commercial BCA protein assay (Pierce Chemical Co, Rockford, IL) with bovine serum albumin, fraction V as the standard.

2.4. Purification of ATH

Purification of ATH was carried out essentially using the procedure of Mittenbühler and Holzer [16]. Modifications are described below:

Crude extract: SEY6210/pDAT1.9 yeast cells were grown in YPD to an $A_{600} \sim 11.0$, harvested by centrifugation (300 g wet weight from 12 l of culture medium), washed twice with buffer A (40 mM sodium citrate, pH 4.5, 1 mM EDTA), and resuspended in 550 ml lysis buffer (440 ml buffer A, 110 ml glycerol) containing 1 mM PMSF. Cells were disrupted using an Eaton press (American Instrument Co., Silver Spring, MD) at 20 000 lb/inch^2.

Hydrophobic interaction chromatography: Fractions with highest activity were pooled, and desalted by passage through a Sephadex G-25 column (1.5 cm × 100 cm).

Gel filtration chromatography: The protein solution obtained from the previous step was loaded on a Superose 12 gel filtration column (HR 10/30; Pharmacia, Piscataway, NJ), equilibrated with buffer B (40 mM sodium citrate, 1 mM EDTA, pH 4.5). Acid trehalase was eluted from the column using buffer B. Fractions with highest ATH activity were pooled, stored on ice and precipitated with 80% ethanol (final concentration) for 12–18 h at 0°C. The precipitate was dissolved in distilled H_2O.

Preparative isoelectric focusing: The protein solution (1.0 mg) obtained from the Superose 12 purification step was injected into a prefocused standard Biolyte-filled chamber of a Rotofor preparative isoelectric focusing system (BioRad, Hercules, CA) in a narrow pH gradient range, pH 3.9–5.6. Fractions were simultaneously aspirated into 20 individual test tubes (1 ml/tube) and each fraction was assayed for ATH activity. The Rotofor purification resulted in an apparent decrease in the fold purification due to dilution (Table 1). This step, however, allowed the resolution of the ATH band from a contaminant of lower molecular mass (approx. 33 kDa; Fig. 1, lane 4).

2.5. Deglycosylation by endoglycosidase H

Acid trehalase was denatured at 95°C for 10 min in 0.1 M sodium acetate, pH 5.5, 0.5% SDS, 0.5% β-mercaptoethanol. The denatured protein was then treated with endo H (Boehringer Mannheim Biochemicals, Indianapolis, IN; 0.5 mU/1 µg protein for 2–4 h at 37°C) in endo H buffer (50 mM sodium citrate, pH 5.5).

2.6. Proteinase digestion and peptide sequencing

The deglycosylated purified ATH protein was transferred to PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA) and digested with endopeptidase Lys-C. Peptide fragments were resolved by HPLC. Two peptides were sequenced by automated Edman degradation, with subsequent identification of the PTH-amino acids by HPLC.

3. Results

3.1. Purification of acid trehalase

As a first step in the characterization of the vacuolar acid trehalase (ATH), we decided to purify the enzyme and determine if it is the product of the *ATH1* gene. ATH activity copurifies with the secreted enzyme invertase [16]. To reduce the possibility of contaminants in the purification procedure, we initially carried out a small scale preparation utilizing a yeast strain, MDY3 [1], having disruptions in both the *SUC2* and *YGP1* genes (data not shown). After identifying the ATH protein, free of the known invertase and gp37 contaminants, the purification was repeated on a larger scale starting with a strain that overproduced ATH from a multi-copy plasmid (SEY6210/pDAT1.9). Table 1 summarizes the purification of ATH from this strain. We achieved a 37% yield with approx. 600-fold purification. The apparent increase in ATH activity following the first two steps of purification, i.e. the citric acid and ammonium sulfate fractionation steps, may represent ac-

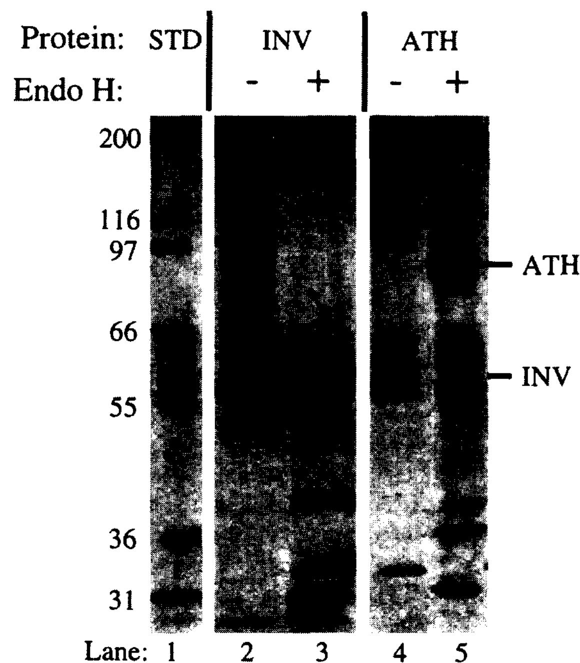

Fig. 1. ATH is a glycoprotein. Purified ATH and commercially purified invertase were deglycosylated with endo H as indicated, run on an SDS polyacrylamide gel and detected by staining with Coomassie Blue. Lanes: 1, molecular weight standards; 2, 35 µg of glycosylated invertase; 3, 35 µg of deglycosylated invertase; 4, 6 µg of Superose 12 purified ATH; 5, 6 µg of Superose 12 purified, deglycosylated ATH. The positions of deglycosylated invertase and deglycosylated ATH are indicated. −, sample without endo H treatment; +, sample treated with endo H.

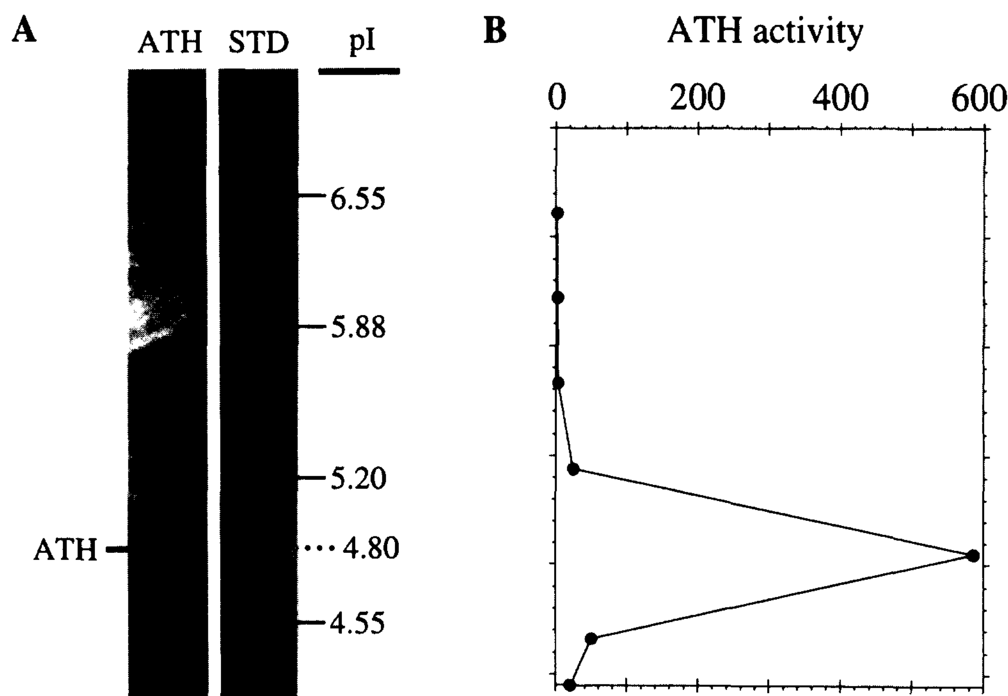

Fig. 2. Isoelectric focusing of acid trehalase. 1.0 μg of ATH from the Rotofor purification step was run on the Pharmacia PhastSystem. The standard protein markers and their p*I* values are: (a) soybean trypsin inhibitor (4.55), (b) β-lactoglobulin A (5.20), (c) bovine carbonic anhydrase B (5.88), (d) human carbonic anhydrase B (6.55). (A) Coomassie Blue-stained IEF gel of ATH and of marker protein. The pH profile of the gel is indicated based on the migration position of the protein standards. The position of ATH is indicated and the dotted line marks the calculated p*I*. (B) An identical sample of ATH was run on the IEF gel. The gel was sliced into sections, homogenized and assayed for ATH activity as described in Section 2. The ATH activity profile of the homogenized gel slices is shown. ATH activity is expressed as nmol glucose released/gel slice per 30 min.

tivated latent activity; cytosolic inhibitors have been characterized for several vacuolar hydrolases [26].

3.2. Properties of the purified acid trehalase

The purified enzyme exhibited a broad smear corresponding to a molecular mass in the range of 143–203 kDa above a sharply defined band (Fig. 1, lane 4). The molecular mass of ATH was estimated to be 210 kDa by high-performance gel filtration (data not shown). This is in agreement with the size of ATH estimated previously by others [16,27]. The purified glycosylated ATH preparation contains an additional band migrating at approx. 33 kDa (Fig. 1). This lower molecular weight band does not fractionate with ATH activity following purification by isoelectric focusing (data not shown). Lane 4 of Fig. 1 represents a pooled set of fractions that include this lower molecular weight species. The pH optimum of the purified enzyme was found to be approx. 4.0 in the crude extract, as well as in the purified protein preparation (data not shown). This pH activity profile also fits well with published data [16,27].

3.3. ATH is a glycoprotein

The migration pattern of the purified enzyme is typical of proteins that undergo heterogeneous glycosylation and suggests that ATH is a glycoprotein. It is worth noting, however, that this type of glycosylation is not seen with any previously characterized vacuolar hydrolases; vacuolar glycoproteins generally undergo limited glycosyl modification, resulting in sharply defined bands on SDS-PAGE gels [25,28–30]. In contrast, secreted proteins including invertase tend to run as a smear under identical conditions ([31]; Fig. 1, lane 2). Incubation of the ATH preparation with Concanavalin A-Sepharose removed all ATH activity from the supernatant (data not shown), further confirming that ATH is a glycoprotein.

To determine the size of the deglycosylated protein, the carbohydrate moieties were removed from the Superose 12-purified ATH preparation using endo H. SDS-PAGE revealed the presence of a doublet at approx. 85 kDa, a 31 kDa species and an additional band migrating at approx. 37 kDa (Fig. 1, lane 5). The band migrating at approx. 31 kDa is probably the deglycosylated form of the 33 kDa contaminant. Analysis of the ATH preparation from the Δ*ygp1* strain showed only the 85 and 31 kDa bands (data not shown), suggesting that the 37 kDa species is the deglycosylated *YGP1* gene product, gp37. While it is possible that the highly glycosylated 85 kDa species corresponds to a copurifying contaminant protein, the lack of additional bands other than gp37 and the 31 kDa protein following deglycosylation suggests that this highly glycosylated protein is ATH.

To ascertain the identity of the doublet at approx. 85 kDa, the ATH preparation was further fractionated on an isoelectric focusing gel (Fig. 2). The ATH preparation was run in two parallel lanes and the gel was divided in half. One half of the gel was stained with Coomassie Blue to allow a determination of the isoelectric point. The remaining half of the gel was cut into thin horizontal pieces, ground up and again divided into two samples. One sample was used to measure ATH activity. The peak ATH activity (Fig. 2A) coincided with a Coomassie Blue stained doublet band migrating in the pH zone of 4.8 (Fig. 2B). An isoelectric point of 4.8 is in close agreement with the previously reported p*I* for ATH [16]. The remaining sample from the homogenized gel slice

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Deduced Athlp:   238 Tyr Glu Asn Ser Thr Asn Pro Ile Asn Ser Ser Glu Ser Phe 251
Purified ATH:        Tyr Glu --- Ser Thr Asn Pro Ile Asn Ser --- Glu --- Phe

Deduced Athlp:   255 Asp Val Ser Ser Asn Ile Tyr Asn Val Ile Leu Thr 266
Purified ATH:        Asp Val Ser Ser Asn Ile Tyr Asn --- Ile --- Thr
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Fig. 3. ATH is the *ATH1* gene product. The deglycosylated purified ATH was subjected to endoproteinase Lys-C digestion and Edman degradation as described in Section 2. The amino acid sequence was determined from an internal peptide of both the upper and lower bands of the ATH doublet (see Fig. 1). The amino acid sequences are shown below the corresponding deduced sequences from the *ATH1* gene. The numbers indicate the position of residues in Ath1p [1]. The upper and lower peptides correspond to amino acid sequences from the upper and lower bands of the doublet, respectively.

corresponding to the peak ATH activity was subjected to endo H treatment. SDS-PAGE analysis revealed a doublet migrating at 85 kDa (data not shown). A similar doublet band of approx. 85 kDa which corresponds to ATH was also seen by Mittenbühler and Holzer after deglycosylation of their purified protein preparation ([9]; Fig. 1). However, they analyzed the 37 kDa protein (now known to represent gp37 [17]) as ATH instead of the 85 kDa band. These results suggest that the 85 kDa deglycosylated species correspond to ATH.

3.4. *Amino acid analysis confirms that ATH1 encodes ATH*

The *ATH1* gene encodes a protein with a predicted molecular mass of 117 kDa. Many vacuolar hydrolases are initially synthesized as larger zymogens that are processed to an active form upon delivery to the vacuole [7]. Similarly, ATH activity is reported to be dependent on the *PEP4* gene product, PrA [11]. To further define the relationship between the *ATH1* gene and the 85 kDa protein(s) in the deglycosylated ATH preparation, we determined the amino acid sequence from internal peptide fragments from each band in the doublet. The doublet bands were resolved on an 8% polyacrylamide gel, transferred to PVDF, cleaved with protease and subjected to Edman degradation as described in Section 2. The sequenced peptides aligned completely with the deduced amino acid sequence from the *ATH1* gene (Fig. 3); the sequences of peptides from the upper and lower bands corresponded to residues 238–251 and 255–266 of the deduced Ath1p sequence, respectively. Both the upper and lower bands from the doublet are derived from *ATH1*, suggesting that the lower species may be a degradation product. Both species showed very similar peptide profiles on HPLC following endoproteinase Lys-C digestion (data not shown), providing a further indication that they are derived from a common polypeptide. Finally, the two bands have almost identical isoelectric points (Fig. 2). The N terminus of the upper band was found to be blocked, making it impossible to determine if there is an N-terminal propeptide cleavage site.

3.5. *Antiserum to Ath1p specifically detects ATH*

As a further test of the relationship between ATH and the *ATH1* gene product, we generated antiserum to a *TrpE-ATH1* hybrid protein as described in Section 2. Crude extracts were prepared from wild-type, *Δath1* and the ATH overexpressing strains of yeast. The extracts were taken through the ammonium sulfate fractionation step and then desalted to partially purify the ATH protein. The samples were resolved by SDS-PAGE and proteins were detected by immunoblot. Antiserum to Ath1p detects a protein of approx. 85 kDa in a wild-type yeast strain but not in an isogenic *Δath1* strain, indicating that the antiserum is specific to ATH (Fig. 4). Overexpression of ATH activity on a multi-copy plasmid results in an increased level of protein detectable with the antiserum to Ath1p, providing additional confirmation that ATH is the *ATH1* gene product.

4. Discussion

The yeast *S. cerevisiae* contains two enzymes capable of degrading the disaccharide trehalose. The gene encoding the neutral cytosolic trehalase, *NTH1* [32], has been cloned and the gene product identified [33]. In contrast, much less is known about the vacuolar acid trehalase. Previous studies of ATH have been hampered by the copurification of this enzyme with other proteins. It is not known why the highly glycosylated proteins invertase and gp37 copurify with ATH. Possibly ATH binds these proteins due to its nature as a glycosidase. Alternatively, ATH may itself be highly glycosylated giving it similar properties with regard to purification. Recently, we identified a gene, *ATH1*, that is required for ATH activity [1]. The lack of a consensus signal sequence cleavage site and the absence of homology to known trehalases, however, made it unclear as to whether *ATH1* was the structural gene for ATH, or whether it encoded a regulatory protein. To clarify the relationship of *ATH1* to ATH we purified the vacuolar acid trehalase (ATH) activity. After purification of ATH, we determined the partial amino acid se-

Table 1
Purification of acid trehalase

Step	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1. Crude extract	620	2728	0.2	1	100
2. Citric acid	590	3737	2	10	137
3. Ammonium sulfate	700	4317	23	115	158
4. Spherogel	102	2400	109	545	88
5. Sephadex G-25	105	1866	110	550	68
6. Superose 12	8	1083	119	595	40
7. Rotofor	10	1020	113	565	37

quence of the trehalase protein. We found a perfect match between the sequences in ATH and deduced sequences in *ATH1*. In addition, antiserum raised to a *TrpE-ATH1* hybrid protein specifically recognized the purified ATH protein, further indicating that ATH is the *ATH1* gene product.

ATH activity has been localized to the vacuole [27] and is reported to be dependent on the *PEP4* gene product PrA [11]; there is a 4-fold reduction in ATH activity in a Δ*pep4* strain. We have observed a similar reduction in ATH activity in *pep4* strains (data not shown). The reported *PEP4* dependence of ATH activity [11] suggests the presence of a propeptide that is cleaved in the vacuole by PrA or a PrA-dependent hydrolase such as PrB. This is consistent with the fact that the 85 kDa ATH is substantially smaller than the potential 117 kDa protein encoded by the *ATH1* open reading frame. A 30 kDa peptide would be large but is approximately the size of the N-terminal propeptide of the vacuolar hydrolase proteinase B [30]. Because our antiserum to Ath1p only detects ATH in western blots and not by immunoprecipitation, we are not able to carry out a kinetic analysis to directly demonstrate processing of the enzyme. Purification of ATH from a Δ*pep4* strain revealed a band that co-migrated with ATH from a wild-type strain (data not shown). We think it likely, however, that the labile propeptide was degraded during the purification procedure.

The cytosolic neutral trehalase is subject to phosphorylation-dependent activation while ATH is constitutively active [34,35]. This differential regulation is necessary because trehalose lines the plasma membrane where it would be exposed to neutral trehalase; ATH activity is sequestered within the vacuole. The role of ATH in trehalose metabolism is not understood. The enzyme is synthesized as cells exit from logarithmic phase growth [36] at the same time that trehalose accumulates [37]. ATH may play a role in mobilization of trehalose upon return to vegetative growth conditions. Catabolite inactivation of the trehalose synthesizing enzymes occurs in the presence of glucose [38]. Once the synthesizing enzymes are destroyed, the conditions presumably warrant the eventual degradation of trehalose as well, although the timing of these two events has not been correlated.

Another question that remains unanswered is the mechanism by which trehalase gains access to its substrate. Both endocytosis and autophagy are general mechanisms used for vacuolar delivery of extracellular and cytoplasmic constituents, respectively, prior to degradation. In the case of endocytosis, the specific signals that trigger inclusion of proteins into endocytic vesicles are at best partly understood for a limited number of proteins. Trehalose could be internalized from the cell surface and delivered to the vacuole via endocytosis. Autophagy is even less well characterized but could be used for the vacuolar uptake of cytosolic trehalose. A plasma membrane trehalose transporter has been shown to be involved in the extracellular localization of the disaccharide [39]. It is not known whether a similar transport mechanism exists in the vacuole membrane. Whichever the case, the signals that might trigger trehalose uptake into the vacuole are not known. Our identification of *ATH1* as the gene encoding ATH allows us to address questions of trehalose metabolism by focusing on the biosynthesis of the vacuolar acid trehalase and the regulation of the *ATH1* gene.

After preparing this manuscript, a paper from the group of H. Holzer was published [21] which showed ATH as necessary for utilization of extracellular trehalose and demonstrated a gene dosage effect of the *ATH1* gene for ATH activity and growth on trehalose. These data provide the first information on the physiological role of vacuolar acid trehalase.

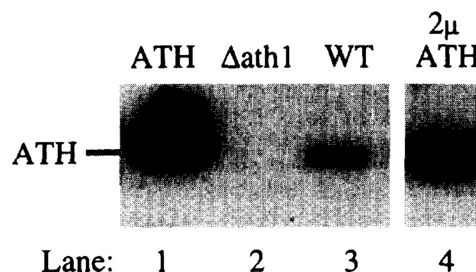

Fig. 4. Antiserum to Ath1p specifically detects ATH. ATH was purified through step 3 of the purification procedure as described in Section 2 (see Table 1) from the Δ*ath1* (MDY3), wild-type (SEY6210) and *ATH1*-overproducing (SEY6210/pDAT1.9) strains. Following desalting, the ATH preparations were deglycosylated with endo H. Partially purified, deglycosylated ATH was subjected to SDS PAGE, transferred to PVDF membrane and probed with antiserum to Ath1p. Signals were detected using chemiluminescence. Lanes: 1, 0.5 μg purified ATH from the Rotofor step; 2, 5 μg of protein from the Δ*ath1* strain; 3, 5 μg of protein from the wild-type strain; 4, 5 μg of protein from the overproducing strain.

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