

Purification and Characterization of Acid Trehalase from the Yeast *suc2* Mutant*

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Acid trehalase was purified from the yeast *suc2* deletion mutant. After hydrophobic interaction chromatography, the enzyme could be purified to a single band or peak by a further step of either polyacrylamide gel electrophoresis, gel filtration, or isoelectric focusing. An apparent molecular mass of 218,000 Da was calculated from gel filtration. Polyacrylamide gel electrophoresis of the purified enzyme in the presence of sodium dodecyl sulfate suggested a molecular mass of 216,000 Da. Endoglycosidase H digestion of the purified enzyme resulted after sodium dodecyl sulfate gel electrophoresis in one distinct band at 41,000 Da, representing the mannose-free protein moiety of acid trehalase. The carbohydrate content of the enzyme was 86%. Amino acid analysis indicated 354 residues/molecule of enzyme including 9 cysteine moieties and only 1 methionine. The isoelectric point of the enzyme was estimated by gel electrofocusing to be approximately 4.7. The catalytic activity showed a maximum at pH 4.5. The activity of the enzyme was not inhibited by 10 mM each of HgCl_2 , EDTA, iodoacetic acid, phenanthroline chloride or phenylmethylsulfonyl fluoride. There was no activation by divalent metal ions. The acid trehalase exhibited an apparent K_m for trehalose of 4.7 ± 0.1 mM and a V_{max} of 99 μmol of trehalose $\text{min}^{-1} \times \text{mg}^{-1}$ at 37 °C and pH 4.5. The acid trehalase is located in the vacuoles. The rabbit antiserum raised against acid trehalase exhibited strong cross-reaction with purified invertase. These cross-reactions were removed by affinity chromatography using invertase coupled to CNBr-activated Sepharose 4B. Precipitation of acid trehalase activity was observed with the purified antiserum.

Trehalose, a nonreducing disaccharide (α -D-glucopyranosyl(1 $\rightarrow$ 1)- α -D-glucopyranoside) was first isolated by Wiggers in 1832 (1). Since then it has been found in a wide variety of organisms including yeast, fungi, bacteria, plants, insects, and other invertebrates. In *Saccharomyces cerevisiae*, trehalose is one of the major storage carbohydrates, accounting for 1% to more than 23% of the dry weight of the cells, depending on the growth conditions and the stage of the life cycle (2). Interestingly, the large pools of trehalose remain constant in cells starved by incubation in water even though these cells contain high activities of trehalase, the trehalose-hydrolyzing enzyme, first described in yeast by Fischer (3). Such observations led to the postulation of an intracellular compartmen-

talization of trehalase and its substrate in yeast (4, 5). Further investigations by Souza and Panek (6) indicated that after disruption and centrifugation of protoplasts, the trehalase was found in the soluble fraction, whereas trehalose remained in the corresponding sediment. From these results they concluded that the substrate, trehalose, was separated from the enzyme through its binding to special sites on the cytoplasmic membranes. In 1974, van der Plaats and van Solingen (7) reported that the trehalase activity increased substantially and almost instantaneously upon the initiation of growth of stationary yeast cultures. This appeared to involve the activation of a preexisting trehalase zymogen by a cAMP-dependent protein kinase (8). In 1982, Wiemken and co-workers (9, 10) demonstrated localization of the trehalase zymogen in the cytosol and localization of a second, permanently active trehalase in the vacuoles. Londesborough and Varimo (11) separated these two activities by protein fractionation and reported their different physical and catalytic properties using partially purified enzymes. This presented clear evidence for the existence of two different activities in yeast, one assayed at pH 5 that was confined to the vacuoles and the other with a maximum activity at pH 7 that was located in the cytosol and interconverted by phosphorylation/dephosphorylation (8, 12, 13). In the present paper, the vacuolar trehalase has been purified to SDS¹ gel homogeneity and characterized. This enzyme is designated "acid trehalase" due to its maximal activity at pH 4.5. In contrast, the cytosolic trehalase exhibiting a pH optimum at 7.0 is designated "neutral trehalase."

MATERIALS AND METHODS

Reagents

Auxiliary enzymes and biochemicals were purchased from Boehringer (Mannheim, Federal Republic of Germany) and Merck (Darmstadt, FRG). Orcinol and 2,3,5-triphenyltetrazolium chloride were from Sigma (Taufkirchen, FRG). Concanavalin A, CNBr-activated Sepharose CL-4B, protein A-Sepharose CL-4B, PD-10 columns, and Pharmalyte pH 2.5-5.0 were from Pharmacia LKB Biotechnology Inc. (Freiburg, FRG). The Spherogel® TSK phenyl-5PW column for high performance hydrophobic interaction chromatography was from Beckmann (München, FRG).

Organism

The diploid yeast *suc2* deletion mutant 2.64.1 C α producing no external invertase was used in all experiments. In this strain, the SUC2 gene is substituted with the URA3 gene. The strain was a gift from Prof. Dr. Zimmermann and Dr. Stefan Hohmann, Institut für

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¹ The abbreviations used are: SDS, sodium dodecyl sulfate; Mes, 4-morpholineethanesulfonic acid; Endo H, endo- β -N-acetylglucosaminidase H; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I, liter.

Genetik, TH Darmstadt, FRG. Bakers' yeast was obtained from Bako, Darmstadt, FRG.

Growth Conditions

suc2 cells, cultivated in YEPD medium (1% Bacto yeast extract, 2% bacto-peptone, 50 mg/l adenine, 50 mg/l uracil, and 2% glucose) for 48 h at 30 °C (stationary phase), were used for the purification of the enzyme.

Enzyme Assays

Acid trehalase was assayed by incubating 30 μ l of extract with 270 μ l of 200 mM sodium citrate, pH 4.5, containing 1 mM EDTA and 114 mM trehalose. After 15 min, the reaction was stopped by adding 50 μ l of the incubation mixture to 950 μ l of boiling H₂O and heating up to 95 °C for 3 min. The controls were stopped immediately. After centrifugation at 10,000 $\times$ g for 3 min at 4 °C, the glucose concentration of the supernatant liquid was determined enzymatically by the glucose oxidase-Perid-II method using glucose oxidase and peroxidase (14). The measured activity was linear with respect to the amount of enzyme (data not shown). Invertase was assayed in the same way as acid trehalase, except that 114 mM sucrose was used instead of 114 mM trehalose. For studies on Me²⁺ effects, EDTA was omitted from the incubation mixture. Proteinase B was assayed with azocoll as substrate as described by Sahelki and Holzer (15). α -Mannosidase was tested with *p*-nitrophenyl- α -D-mannopyranoside according to van der Wilden *et al.* (16). α -Glucosidase was measured as described by Halvorson (17) and Glc-6-P dehydrogenase was assayed as in Ref. 18.

Definition of Units

One unit of acid trehalase is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μ mol of trehalose/min at 37 °C and pH 4.5. One unit of invertase catalyzes the hydrolysis of 1 μ mol of sucrose/min at 37 °C and pH 4.5.

Carbohydrate Analysis

The neutral sugar content of acid trehalase was determined by the orcinol method as described by Winzler (19) using mannose as standard. The absorption was measured at 505 nm instead of 540 nm (20).

Amino Acid Analysis

Amino acid analysis was carried out with a Biotronik LC 6000 amino acid analyzer as previously described (21, 22). Half-cysteine was measured as cysteic acid after performic acid oxidation (23). The tryptophan content in the protein was determined spectrophotometrically according to Edelhoch (24).

Absorption Spectra

Spectra in the range from 220 to 500 nm were recorded with a Lambda 7 Perkin-Elmer spectrophotometer equipped with a Perkin-Elmer Lambda computer.

Protein Determination

Protein was determined by the method of Bradford (25) using the commercial Bio-Rad protein assay with crystalline bovine serum albumin as standard.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out at pH 8.3 on 10% gels in the presence of SDS as described by King and Laemmli (26). Gels were stained for protein with Coomassie Brilliant Blue R-250 and destained by diffusion. Isoelectric focusing using Pharmalyte pH 2.5–5.0 was performed in 10-cm gel rods according to Wrigley (27) except that the cathode buffer was 10 mM Hepes and the anode buffer was 10 mM iminodiacetic acid. The 5% gels contained 6.5% carrier ampholyte. The pH was measured by cutting one gel into 2-mm slices and eluting each slice with 750 μ l of water. Activity staining was done by the method of Gabriel (28) detecting the released glucose.

Antiserum Preparation and Purification

A rabbit was immunized against acid trehalase and boosted 4 and 8 weeks later. The antiserum was titrated and checked for cross-reactivity by Ouchterlony analysis (29) and Western blot as described by Schäfer *et al.* (30). Nonspecific reactivity with other high-mannose

glycoproteins of yeast was removed by affinity chromatography using invertase coupled to CNBr-activated Sepharose CL-4B.

Isolation of Vacuoles

Cells were grown in YEPD medium for 18 h at 30 °C. Spheroplast formation, lysis of spheroplasts with DEAE-dextran, and purification of vacuoles were performed as described by Mechler *et al.* (31). Enrichment of vacuoles as determined by vacuolar enzyme activity was 60- to 95-fold.

Purification of Acid Trehalase from the Yeast *suc2* Mutant (All Operations Were Performed at 4 °C)

1. *Crude Extract*—Yeast cells (see "Growth Conditions") were harvested (120 g wet weight), washed twice with buffer A (40 mM sodium citrate, 1 mM EDTA, pH 6.5) by centrifugation (2000 $\times$ g for 5 min), and suspended to 50% (wet weight/volume) in the same buffer. Cells were disrupted by passing the suspension three times through a French pressure cell. The supernatant resulting from centrifugation at 10,000 $\times$ g for 60 min is referred to as the crude extract.

2. *Acid Fractionation*—Citric acid (2 M) was added with stirring and with ice bath cooling to adjust the pH of the crude extract to 4.0. Precipitated protein was removed by centrifugation at 10,000 $\times$ g for 60 min.

3. *Ammonium Sulfate Fractionation*—Solid ammonium sulfate (43 g) was added to 100 ml of supernatant from the acid fractionation with stirring for 30 min at 0 °C. Precipitated protein was removed by centrifugation at 10,000 $\times$ g for 45 min.

4. *Hydrophobic Interaction Chromatography with Phenyl-Sepharose CL-4B*—The supernatant of the ammonium sulfate fractionation (150 ml) was applied to a column of phenyl-Sepharose CL-4B (2.5 $\times$ 11 cm) equilibrated with buffer B (40 mM sodium citrate, 1 mM EDTA, pH 4.5, and 43 g of solid ammonium sulfate added to 100 ml, equivalent to 69% saturation at 0 °C). The column was washed with 400 ml of buffer B, and the enzyme was eluted with a linear decreasing gradient of 69%, pH 4.5, to 0%, pH 6.5, ammonium sulfate saturation. The enzyme eluted at an ionic strength corresponding to 200 microSiemens.

5. *Hydrophobic Interaction Chromatography Using the High Performance Liquid Chromatography Column Spherogel® TSK Phenyl-5PW*—The peak fractions from the phenyl-Sepharose CL-4B column were collected and 30% (w/v) solid ammonium sulfate was added. The fractions were then passed through a Spherogel® TSK phenyl-5PW column (21.5 mm $\times$ 15 cm) equilibrated with buffer B and were washed with an additional 100 ml of the same buffer. Acid trehalase was eluted from the hydrophobic column by applying a linear decreasing gradient of ammonium sulfate from 69 to 0% saturation using buffer B and H₂O as final eluant. Fractions with highest activity were pooled and either stored at 4 °C or passed through a PD-10 column and then precipitated with 80% ethanol (final concentration) for 24 h at 0 °C. The precipitate was centrifuged and stored at –20 °C. For further use, the precipitate was dissolved in water.

Crude Extract from Bakers' Yeast

Yeast cells were washed three times with 40 mM sodium citrate buffer, pH 6.5, containing 1 mM EDTA and suspended to 50% (wet weight/volume) in the same buffer. Disruption and centrifugation conditions were as described under "Purification of Acid Trehalase from the Yeast *suc2* Mutant, 1. Crude Extract."

RESULTS AND DISCUSSION

As separation of invertase from acid trehalase was found to be extremely difficult and time consuming, trehalase was purified from the yeast *suc2* deletion mutant, which contains no external invertase. The five-step procedure is summarized in Table I. An approximately 7,000-fold purification with a yield of 38% was obtained. SDS-polyacrylamide gel electrophoresis of the crude extract and of aliquots from steps 2–5 of the purification procedure is shown in Fig. 1. The purified enzyme resulting from step 5 was applied in its native form to high performance gel filtration using a Superose 12 HR 10/30 column. A molecular mass of 218 kDa was estimated (Fig. 2). SDS-polyacrylamide gel electrophoresis resulted in a single broad band ranging from 167,000 to 265,000 Da; from this an

TABLE I
Purification of acid trehalase from the yeast *suc2* mutant

The purification was performed as described under "Materials and Methods." Starting with 120 g, wet weight, of yeast cells, about 500 μ g of purified trehalase were obtained in 7 days.

Step	Volume	Total activity	Specific activity	Purification	Yield	Invertase/ trehalase
	ml	units	units/mg	-fold	%	
1. Crude extract	153	130	0.0138	1	100	0.3
2. Citric acid	133	122	0.115	8	94	0.3
3. Ammonium sulfate	151	117	2.04	148	90	0.25
4. Phenyl-Sepharose chromatography	250	102	9.10	659	80	0.03
5. Spherogel TM TSK phenyl 5PW chromatography	12	49.8	99	7174	38	0.003

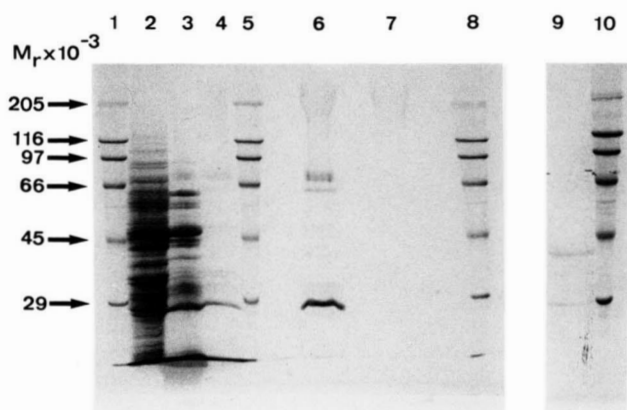


FIG. 1. SDS-polyacrylamide gel electrophoresis of acid trehalase preparations. Samples obtained after different purification steps (see Table I) were solubilized and subjected to SDS-polyacrylamide gel electrophoresis (see "Materials and Methods"). Lane 1, marker proteins: myosin ($M_r = 205,000$), β -galactosidase ($M_r = 116,000$), phosphorylase *b* ($M_r = 97,400$), albumin ($M_r = 66,000$), ovalbumin ($M_r = 45,000$), carbonic anhydrase ($M_r = 29,000$). Lane 2, 150 μ g of protein from crude extract. Lane 3, 60 μ g of protein after acid precipitation (step 2). Lane 4, 60 μ g of protein after ammonium sulfate precipitation (step 3). Lane 5, marker proteins as in lane 1. Lane 6, 55 μ g of protein after phenyl-Sepharose chromatography (step 4). Lane 7, 20 μ g of protein after Spherogel[®] TSK phenyl-5PW chromatography (step 5). Lane 8, marker proteins as in lane 1. Lane 9, acid trehalase (10 μ g) treated with endo H (24 h); the protein band at 29,000 Da represents the endoglycosidase H. Lane 10, marker proteins as in lane 1.

average molecular mass of 216 kDa was calculated for acid trehalase. The high carbohydrate content of yeast acid trehalase is probably responsible for the broad band in SDS-gel electrophoretograms, as it is for external invertase (32, 33). SDS-gel electrophoresis with and without dithiothreitol showed the same molecular weight, indicating that the native enzyme is a monomer lacking intermolecular disulfide bridges. A molecular mass of 215 kDa, which is in good agreement with the values presented here, was estimated by Londesborough and Varimo (11) for a partially purified enzyme preparation by Sephadex G-150 gel filtration. Dependence of purified acid trehalase activity on the pH is shown in Fig. 3. Maximal activity was observed at pH 4.5. A similar pH optimum was obtained with a crude extract from bakers' yeast (Fig. 3) and from stationary *suc2* mutant cells (data not shown). The vacuolar pH, which is reported to be about 5.5–5.9 (34, 35), and the optimal pH of acid trehalase are both in an acid range. Earlier investigations under different conditions (11, 36, 37) showed pH optima between 4 and 5.7. EDTA (6.5 mM), in the tested pH range from 2.5 to 7.7, had no effect on the acid trehalase but completely inhibited the neutral

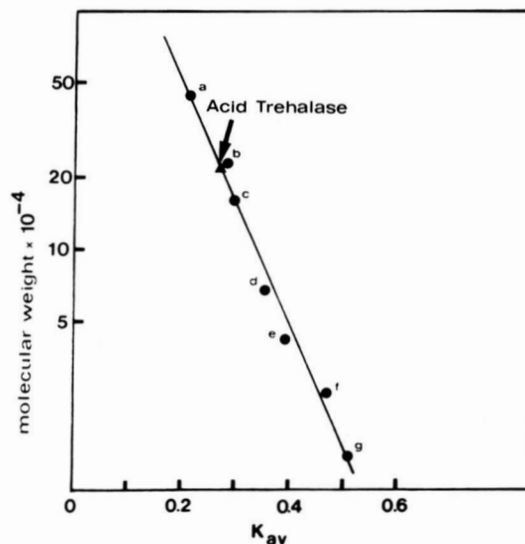


FIG. 2. Estimation of the molecular mass of the purified acid trehalase by comparison with marker proteins in high performance gel filtration. 6 μ g of purified enzyme (Table I, step 5, specific activity 99 units/mg of protein) were passed through a Sepharose 12 HR 10/30 column (1.0 $\times$ 30 cm) equilibrated with 100 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl. Flow rate was 30 ml/h. Fractions of 0.5 ml each were collected. The column was calibrated with (a) ferritin ($M_r = 440,000$); (b) catalase ($M_r = 232,000$); (c) aldolase ($M_r = 158,000$); (d) bovine serum albumin ($M_r = 67,000$); (e) ovalbumin ($M_r = 43,000$); (f) chymotrypsinogen a ($M_r = 25,000$); (g) ribonuclease ($M_r = 13,700$); $K_{av} = (V_e - V_0)/(V_i - V_0)$. Position of the marker proteins was determined by measuring the absorption of 280 nm, and the position of acid trehalase was determined by measuring enzymatic activity.

trehalase activity of the extract from bakers' yeast (Fig. 3), as also shown earlier by Londesborough and Varimo (11). In the absence of EDTA, a second pH optimum was observed at around pH 7 (Fig. 3). This peak which corresponds to the neutral trehalase activity was represented only by a shoulder when using crude extract from *suc2* mutant (data not shown).

The acid trehalase was assayed for substrate specificity at pH 4.5 using the purified enzyme. Acid trehalase showed a high specificity for trehalose. Other disaccharides such as sucrose, maltose, lactose, cellobiose, melibiose, and the trisaccharide raffinose showed essentially no detectable activity (less than 0.3% of the activity measured with trehalose). Kelly and Catley (37) showed that their partially purified acid trehalase exhibits no action on maltose, sucrose, raffinose, cellobiose, starch, glycogen, glucose 6-phosphate and glucose 1-phosphate. Panek and Souza (36) reported specificity only for trehalose and raffinose among several oligosaccharides. This latter observation is in contrast to the findings of Kelly and Catley (37) and to our observations using the purified

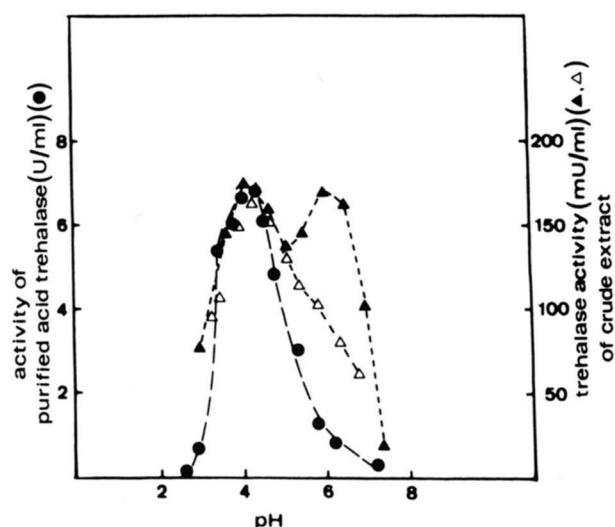


FIG. 3. Dependence of trehalase activity on pH. The activity in a crude extract of bakers' yeast (milliunits/ml) was measured in test mixtures containing 114 mM trehalase, 30 mM citric acid/ Na_2HPO_4 (Mc Ilvaine buffer) without (Δ) and with ($\bullet$) 6.5 mM EDTA as described under "Materials and Methods." The activity of the purified acid trehalase (units/ml) was measured in test mixtures containing 114 mM trehalose, 30 mM citric acid/ Na_2HPO_4 ($\bullet$).

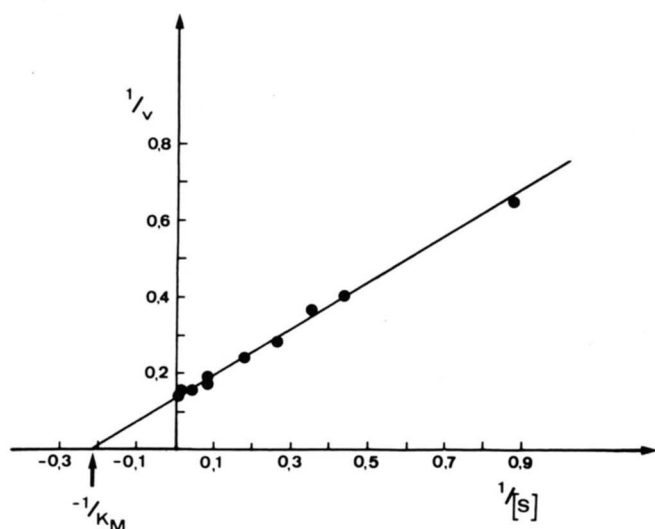


FIG. 4. Determination of K_m of purified acid trehalase using Lineweaver-Burk plot. Acid trehalase activity was assayed using increasing concentrations of trehalose as substrate. Assay conditions were described under "Materials and Methods." The S and K_m values are expressed as mM; V values and $V_{\max}$ are $\mu\text{mol} \times \text{min}^{-1} \times \text{ml}^{-1}$.

enzyme. Our results indicate that the acid trehalase is highly specific for the $\alpha 1\text{-}\alpha 1$ bond.

The K_m for trehalose was 4.7 ± 0.1 mM at pH 4.5, with a corresponding $V_{\max}$ of $99 \mu\text{mol} \times \text{min}^{-1} \times \text{mg}^{-1}$ (Fig. 4). Panek and Souza (36) found a K_m of 0.4 mM at pH 5.6 while a K_m of 0.5 mM at pH 5.5 was reported by Kelly and Catley (37). Londesborough and Varimo (11) determined a K_m for the vacuolar trehalase of 1.4 mM at pH 5.0, 5.8, and 6.5 in 50 mM Mes/KOH containing 100 mM KCl. The different test systems and strains used for the determination of K_m seem to be responsible for the discrepant values obtained.

The amino acid analysis of acid trehalase (Table II) shows that the enzyme contains 10% aromatic residues and 51% polar residues, which is common for a soluble protein (38). Acid trehalase contains 3% tryptophan and 43% hydrophobic

TABLE II

Amino acid composition of acid trehalase from *suc2* mutant

Amino acid	HCl			Average or extrapolated integer
	24 ^a	48 ^a	96 ^a	
<i>residues/molecule^b</i>				
Aspartic acid + asparagine	38.50	39.95	40.88	40
Threonine	24.36	23.02	22.39	26 ^c
Serine	38.90	32.57	28.70	44 ^c
Glutamic acid + glutamine	18.24	20.70	21.56	20
Proline	26.42	26.38	25.17	26
Glycine	33.62	34.60	36.08	35
Alanine	33.08	33.91	35.61	34
Valine	26.63	29.19	28.42	29 ^d
Methionine	1.27	1.21	1.29	1
Isoleucine	20.00	20.94	21.81	22 ^e
Leucine	21.72	21.97	23.88	24 ^e
Tyrosine	8.47	8.34	8.43	8
Phenylalanine	9.59	8.92	8.55	9
Lysine	13.25	12.95	12.58	13
Histidine	4.10	4.18	4.29	4
Arginine	4.17	3.28	2.47	3
Half-cysteine	8.64	8.84	9.01	9 ^{f,g}
Tryptophan	7.28 ^h			7 ^h
Total				354

^a Time of hydrolysis in hours.

^b Values obtained are based on a molecular mass of 41,000 Da, corresponding to the protein moiety of acid trehalase after endo H treatment.

^c Extrapolated to zero hydrolysis time.

^d Values from 48/96-h hydrolysis only are considered.

^e Values from 96-h hydrolysis only are considered.

^f Values from 24-h hydrolysis only are considered.

^g Determined as cysteic acid according to Ref. 23.

^h Determined spectrophotometrically according to Ref. 24.

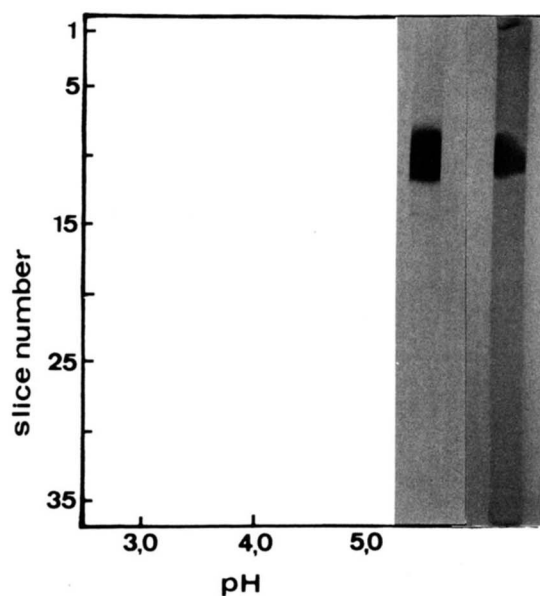


FIG. 5. Isoelectric focusing of acid trehalase. Twenty μg of purified acid trehalase (specific activity 99 units/mg) were applied onto each disc gel (0.6×8.5 cm). After focusing at 500 V for 5 h, pH was measured by cutting one gel into 2-mm-thick slices and extracting with water. For determination of trehalase activity, another gel was stained for activity as described under "Materials and Methods" (left-hand gel). Afterwards, the same gel was stained for protein (right-hand gel). An isoelectric point of 4.7 was estimated.

TABLE III

Distribution of acid trehalase activity and marker proteins in spheroplast and vacuole preparations

The specific activities in the spheroplast lysate are set to be 1. The protein content of the vacuole preparation was 0.275 mg/ml. The spheroplast lysate contained 8.9 mg protein/ml. Protein determination was performed as described under "Materials and Methods."

	Specific activity in purified vacuoles related to spheroplast lysate = 1
Cytosolic markers	
Glucose-6-P dehydrogenase	0.38
α -Glucosidase	0.55
Vacuolar markers	
α -Mannosidase	64
Proteinase B	91
Acid trehalase	93

amino acids. Only one methionine was found among 354 amino acids. The molecular mass of 41,000 Da, obtained after endoglycosidase H treatment (see Fig. 1), was used for calculation of amino acid residues per molecule. Maximal absorption of purified acid trehalase was observed at 276 nm. The molar extinction coefficients calculated for acid trehalase where at $\epsilon_{280} = 98.4 \text{ l} \times \text{mmol}^{-1} \times \text{cm}^{-1}$ and at $\epsilon_{276} = 104.3 \text{ l} \times \text{mmol}^{-1} \times \text{cm}^{-1}$ (protein was calculated from amino acid

analysis). Acid trehalase contains 86% neutral sugars (w/w), as determined with the orcinol-sulfuric acid assay (19, 20) using mannose as a standard. Using the same method, the carbohydrate content of external invertase was determined to be 49.4% which is in agreement with data from Neumann and Lampen (39). Endo H digestion of purified, SDS-denatured acid trehalase in the presence of phenylmethylsulfonyl fluoride for 1 h resulted in two distinct bands after SDS-gel electrophoresis at 41,000 and 100,000 Da. Digestion with Endo H for 2, 4, and 8 h resulted in increasing conversion of the 100-kDa to the 41-kDa form with time. Endo H digestions of 24 h or more resulted in the complete absence of the 100 kDa form, leaving only the 41-kDa mannose-free protein moiety (Fig. 1). Similar results were reported after 20-h incubation of denatured external yeast invertase with Endo H, providing a 62,000-Da band (40, 41) containing no residual oligosaccharides. The purified acid trehalase binds very strongly to concanavalin A. 38% of the applied activity could be eluted with 40 mM sodium citrate, pH 6.0, containing 0.5 M NaCl and 0.5 M methyl- α -D-mannopyranoside (data not shown). Londeborough and Varimo (11), using a dialyzed protamine supernatant, reported a recovery in the collected eluate from a concanavalin A column of 39%.

The susceptibility of the acid trehalase to classical inhibitors was tested using the assay described under "Materials

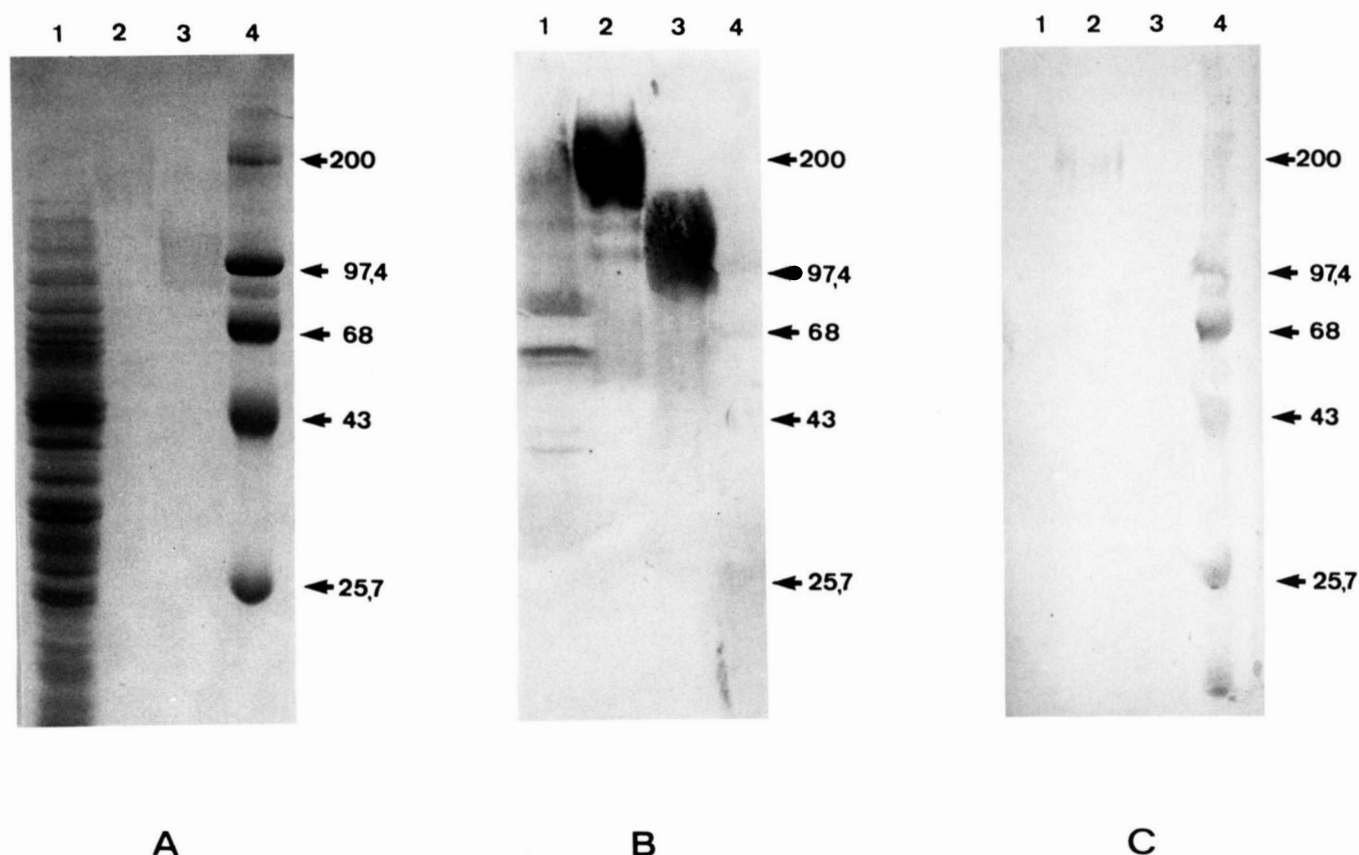


FIG. 6. Comparison by immunoblotting of the acid trehalase antiserum cross-reactivity before and after purification. 180 μ g of protein from crude extract (lane 1), 20 μ g purified acid trehalase (lane 2), 100 μ g of lyophilized, commercial invertase (lane 3), and prestained marker proteins (lane 4) were submitted to SDS-gel electrophoresis and stained with Coomassie Blue (A). The same samples were applied to an SDS gel, run under identical conditions, and blotted onto nitrocellulose sheets. The blotted sheets were incubated with 10 μ l of crude (B) and 20 μ l of purified antiserum (see "Materials and Methods") (C). For C, only 1 μ g of purified acid trehalase instead of 20 μ g was applied to the gel to demonstrate the sensitivity and specificity of the purified antiserum. By reaction with chloronaphthol, the peroxidase-conjugated goat anti-rabbit IgG-bound antibodies were visualized. Numbers indicate the molecular mass standards, in kilodaltons, including myosin (H-chain), phosphorylase b, bovine serum albumin, ovalbumin, and α -chymotrypsinogen.

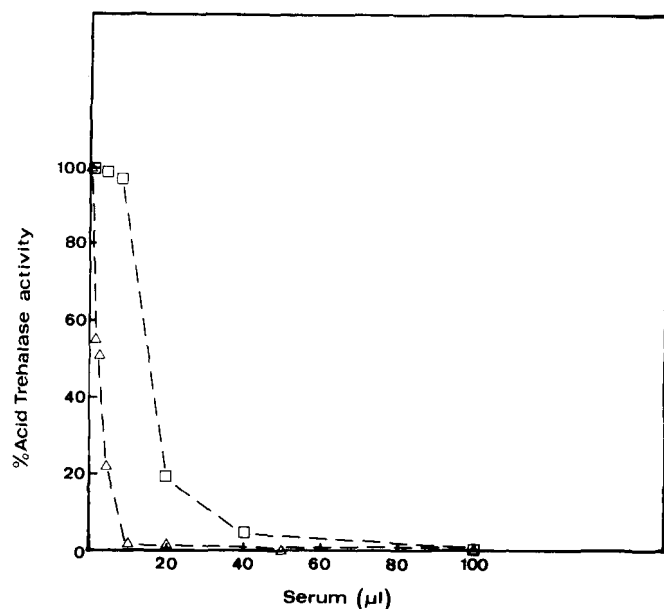


FIG. 7. Immunoprecipitation of acid trehalase activity. Affinity chromatography-purified antiserum and -purified acid trehalase (see Table I) were incubated for 2 h at 25 °C with (Δ) and without (□) protein-A-Sepharose. The precipitated material was removed by centrifugation, and the supernatant was tested for acid trehalase activity as described under "Materials and Methods."

and Methods." Except for 10 mM HgCl_2 , which reduced the activity by about 30%, none of the other substances tested (0.1 and 10 mM of each, EDTA disodium salt, iodoacetic acid, *o*-phenanthroline chloride, phenylmethylsulfonyl fluoride and 0.1 mM HgCl_2) showed a significant effect on the activity of the purified acid trehalase. These results suggest that the active site of the enzyme contains no sulfhydryl, serine, or threonine hydroxyl groups and that its activity is independent of bivalent metal ions. Incubation of the purified enzyme for 1 and 5 h with 0.1 and 10 mM of each inhibitor, prior to determining its activity, also had no significant effect on the acid trehalase activity. The only exception was 10 mM HgCl_2 , which after 1- and 5-h incubations caused 36 and 99% inhibition, respectively. The acid trehalase activity was not altered by replacing the 1 mM EDTA in the assay mixtures with 0.1 or 10 mM CaCl_2 , MnCl_2 , MgCl_2 , or ZnSO_4 . Similarly, preincubation of the enzyme with 0.1 and 10 mM CaCl_2 , MnCl_2 , MgCl_2 , or ZnSO_4 (prior to determining its activity) also had no effect. Similar results were reported by Londesborough (11) with partially purified acid trehalase.

The isoelectric point of purified acid trehalase (99 units/mg) was measured by isoelectric focusing in polyacrylamide gels in a pH range of 2.5–5.0 (Fig. 5). An isoelectric point of 4.7 was determined using activity and protein staining. Identical positions of stained protein and trehalase activity demonstrate that the single visible protein band is responsible for the enzymatic activity. The pI of acid trehalase is in the same range as the isoelectric points of three other vacuolar enzymes from yeast: proteinase A (pI = 4.4) (42), carboxypeptidase Y (pI = 3.6) (43), and aminopeptidase I (pI = 4.7) (44). An acid pI value of acid trehalase is also in accord with the results of Londesborough and Varimo (11).

Subcellular fractionation of lysed spheroplasts by density gradient centrifugation was used to determine the subcellular localization of acid trehalase in yeast. Earlier observations (9) that acid trehalase is confined to the vacuoles were confirmed as shown in Table III; an additional periplasmic localization, however, cannot be excluded.

Immunodiffusion after Ouchterlony (not shown) and Western blot (Fig. 6) analysis showed the antiserum raised against acid trehalase in a rabbit to be reactive not only with the acid trehalase, but also with external invertase and with other glycoproteins in the crude extract. This cross-reactivity was removed completely by affinity chromatography with external invertase coupled to CNBr-activated Sepharose in order to bind the high mannose-specific components (45) of the polyclonal antiserum. Incubation of purified acid trehalase with purified antiserum resulted in complete precipitation of the enzyme activity (Fig. 7).

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REFERENCES

- Wiggers, H. A. L. (1832) *Ann. Pharm. (Pozna)* **1**, 129–182
- Lillie, S. H., and Pringle, J. R. (1980) *J. Bacteriol.* **143**, 1384–1394
- Fischer, E. (1895) *Ber. Dtsch. Chem. Ges.* **28**, 1429–1438
- Myrbäck, K., and Ortenblad, B. (1937) *Biochem. Z.* **291**, 61–69
- Avigad, G. (1965) *Biochem. J.* **97**, 715–722
- Souza, N. O., and Panek, A. D. (1968) *Arch. Biochem. Biophys.* **125**, 22–28
- van der Plaats, J. P. (1974) *Biochem. Biophys. Res. Commun.* **56**, 580–587
- van Solingen, P., and van der Plaats, J. P. (1975) *Biochem. Biophys. Res. Commun.* **62**, 553–560
- Keller, F., Schellenberg, M., and Wiemken, A. (1982) *Arch. Microbiol.* **131**, 298–301
- Wiemken, A., and Schellenberg, M. (1982) *FEBS Lett.* **150**, 329–331
- Londesborough, J., and Varimo, K. (1984) *Biochem. J.* **219**, 511–518
- Uno, I., Matsumoto, K., Adachi, K., and Ishikawa, T. (1983) *J. Biol. Chem.* **258**, 10867–10872
- Ortiz, C. H., Maia, J. C. C., Tenan, M. N., Braz-Padua, G. R., Mattoon, J. R., and Panek, A. D. (1983) *J. Bacteriol.* **153**, 644–651
- Bernt, E., and Lachernicht, R. (1974) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed) Vol. 2, pp. 1260–1266, Verlag Chemie GmbH, Weinheim, West Germany
- Saheki, T., and Holzer, H. (1974) *Eur. J. Biochem.* **42**, 621–626
- Van der Wilden, W., Matile, P., Schellenberg, M., Meyer, J., and Wiemken, A. (1973) *Z. Naturforsch.* **28c**, 416–421
- Halvorson, H., and Elias, L. (1958) *Biochim. Biophys. Acta* **30**, 28–40
- Löhr, G. W., and Waller, H. D. (1974) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed), 3rd Ed., pp. 673–681, Verlag Chemie GmbH, Weinheim, West Germany
- Winzler, R. J. (1955) *Methods of Biochem. Anal.* **2**, 290–292
- Johansen, P. G., Marshall, R. D., and Neuberger, A. (1960) *Biochem. J.* **77**, 239–247
- Spackman, D. H., Stein, W. H., and Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
- Hamilton, P. B. (1963) *Anal. Chem.* **35**, 2055–2064
- Moore, S. (1963) *J. Biol. Chem.* **238**, 235–237
- Edelhoc, H. (1967) *Biochemistry* **6**, 1948–1954
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- King, J., and Laemmli, U. K. (1971) *J. Mol. Biol.* **62**, 465–477
- Wrigley, C. W. (1971) *Methods Enzymol.* **22**, 559–564
- Gabriel, O., and Wang, S.-F. (1969) *Anal. Biochem.* **27**, 545–554
- Ouchterlony, O. (1962) in *Progress in Allergy* (Kallos, P., and Waksman, B. H., eds) Vol. 6, pp. 30–154, Karger, Basel, Switzerland
- Schäfer, W., Kalisz, H., and Holzer, H. (1987) *Biochim. Biophys. Acta* **925**, 150–155
- Mechler, B., Müller, H., and Wolf, D. H. (1987) *EMBO J.* **6**, 2157–2163

32. Trimble, R. B., and Maley, F. (1977) *J. Biol. Chem.* **252**, 4409–4412
33. Williams, R. S., Trumbly, R. J., MacColl, R., Trimble, R. B., and Maley, F. (1985) *J. Biol. Chem.* **260**, 13334–13341
34. Slavik, J. (1982) *FEBS Lett.* **140**, 22–26
35. den Hollander, J. A., Ugurbil, K., Brown, T. R., and Shulman, R. G. (1981) *Biochemistry* **20**, 5871–5880
36. Panek, A., and Souza, N. O. (1964) *J. Biol. Chem.* **239**, 1671–1673
37. Kelley, P. J., and Catley, B. J. (1976) *Anal. Biochem.* **72**, 353–358
38. Capaldi, R. A., and Vanderkooi, G. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 930–932
39. Neumann, N. P., and Lampen, J. O. (1967) *Biochemistry* **6**, 468–475
40. Trimble, R. B., and Maley, F. (1984) *Anal. Biochem.* **141**, 515–522
41. Trimble, R. B., Maley, F., and Chu, F. K. (1983) *J. Biol. Chem.* **258**, 2562–2567
42. Meussdoerffer, F., Tortora, P., and Holzer, H. (1980) *J. Biol. Chem.* **255**, 12087–12093
43. Aibara, S., Hayashi, R., and Hata, T. (1971) *Agric. Biol. Chem.* **35**, 658–666
44. Metz, G., and Röhm, K. (1976) *Biochim. Biophys. Acta* **429**, 933–949
45. Feizi, T., and Childs, R. A. (1987) *Biochem. J.* **245**, 1–11