Biochimica et Biophysica Acta, 524 (1978) 121—130

BBA 68415

α-D-MANNOSIDASE OF *SACCHAROMYCES CEREVISIAE*

CHARACTERIZATION AND MODULATION OF ACTIVITY

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(Received October 19th, 1977)

Summary

A unique and interesting α-D-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) activity has been isolated from *Saccharomyces cerevisiae*. The enzyme was localized in a crude particulate fraction of the cell extract and was not solubilized by treatment with detergents or high ionic strength NaCl.

The enzyme had a pH optimum of 6.3, K_m 50 μM with *p*-nitrophenyl-α-D-mannopyranoside, and was competitively inhibited by D-mannose (K_i 20 mM). The enzyme is not affected by ethylenediaminetetraacetic acid, a number of different cations, or sulfhydryl reagents. It was inhibited by *p*-chloromercuriphenyl sulfonic acid and this inhibition is prevented by the addition of substrate.

The cellular concentration of α-D-mannosidase is inversely proportional to growth rate, suggesting that the enzyme is under catabolite repression. The level of enzyme was found to increase approx. 8-fold during sporulation. This is apparently due to de novo synthesis, since inhibition of protein synthesis by cycloheximide prevents the increase in enzyme activity.

Introduction

Mannose-containing bio-polymers are major components of yeast cells, comprising the mannan complex of yeast cell wall and the glycoprotein molecules, which are found both inside the cell and secreted into the surrounding medium. Although extensive information has been gained on the synthesis of mannose bio-polymers, there is only limited information on the system responsible for the breakdown of these molecules. The importance of α-mannosidase in the degratory system of higher eukaryotic cells is well known, since a deficiency in the enzyme results in the lethal disease, mannosidosis, which occurs in both human [1] and Angus cattle [2].

α-D-Mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) hydrolyzes the glycosidic linkage binding α-D-mannopyranosyl residues to bio-polymers. Although extracellular enzymes have been characterized from bacteria [3] and fungi [4], only limited information exists on the intracellular α-mannosidases of non-mammalian cells. Maddox [5] observed that crude extracts of baker's yeast contain enzymes, optimally active at pH 4.0, that release reducing sugars when incubated with partially-purified mannan. The only α-mannosidase reported to occur in baker's yeast is a soluble enzyme which has little activity on yeast mannan but attacks *p*-nitrophenyl-α-D-mannopyranoside, exhibiting a K_m of 1.0 mM [6]. As the first stage in our analysis of the glyco-polymer degradation system in yeast, we decided to identify and characterize the various α-mannosidase activities in yeast cells. We report here the identification of a particulate α-D-mannosidase which has a low K_m with respect to *p*-nitrophenyl-α-D-mannopyranoside.

Materials and Methods

Cultivation of the microorganism. Saccharomyces cerevisiae strains SK-1 [7] and AP-1-a/α, AP-1-α/α, and α-131-20 [8] were used throughout this study. SK-1 is homothallic and a rapid and synchronously sporulating strain. AP-1-a/α (ade-2) and AP-1-α/α (ade-2) are isogenic except at the mating type locus (8). AP-1-α/α, unlike AP-1-α/a, cannot sporulate and, therefore, makes and excellent control for the identification of sporulation-specific events. α-131-20 is a haploid strain which requires adenine, uracil and leucine for growth.

Cells were grown in either rich media containing 1% yeast extract (Difco), 1% proteose peptone (Difco), and 1—3% of various carbon sources, or minimal media containing 0.17% (w/v) Difco nitrogen base without amino acids and $(NH_4)_2SO_4$ [9], 25 mM $(NH_4)_2SO_4$, 50 μg/ml of adenine, 0.5% casamino acids, and 1—3% (w/v) of various carbon sources. Cells (500 ml) were grown in a 2-l flask on a rotary shaker (340 rev./min with a 1-inch orbit) at 30°C. Growth was followed by measuring the increase in absorbance at 660 nm.

Yeast cells were sporulated in a manner similar to that described by Roth and Halvorson [10]. Cells were exponentially grown in medium containing 1% yeast extract (Difco), 1% proteose peptone (Difco) and 1% sodium acetate. At a density of approx. $2 \cdot 10^7$ cells/ml, the cells were harvested by centrifugation, washed once with 10 ml 1% NaCl, and resuspended at $2 \cdot 10^7$ cells/ml in a 2-l flask containing 300 ml of 1% potassium acetate. The flasks were incubated as described above. Sporulation was monitored by phase contrast microscopy. Samples of at least 200 cells were counted for the presence of 2—4 spored asci; buds were counted as separate cells.

Preparation of cell free extract. Cells, at a density of $1—3 \cdot 10^7$/ml were rapidly cooled to 5°C by the addition of ice and then harvested by centrifugation at $3300 \times g$ for 5 min in a Sorvall GS-3 rotor. Cells were washed once with cold 20 mM sodium acetate (pH 6.5) and were either assayed immediately or were frozen at —20°C for up to 1 week. Enzyme activity was stable at —20°C for at least 2 weeks.

The cells were then suspended in 5 ml 20 mM sodium acetate (pH 6.5) and 2 mM phenylmethylsulfonyl fluoride in a 12-ml glass tube (Pyrex No. 9825) with 5 g of 0.25 mm diameter, acid-washed glass beads (B. Braun, Apparatebau,

Melsungen No. 54160). The tube was capped, placed in ice and shaken for 5 min with a vertical pounding motion. This treatment resulted in approx. 90% breakage of cells, asci, and spores. The glass beads were allowed to settle for 2 min, the solution was decanted and the beads were washed with 4 ml 20 mM sodium acetate (pH 6.5). The supernatant solutions were combined and centrifuged at 1900 × *g* for 5 min in a Sorvall SS-34 rotor. The crude extract was then assayed for enzyme activity. To measure enzyme solubility, the supernatant solution was then centrifuged at 175 000 × *g* in a Beckman 60Ti rotor for 1 h and the distribution of activity between pellet and supernatant solution was determined.

A particulate pellet was prepared from SK-1 cells for use in kinetic analysis by centrifuging the crude extract at 175 000 × *g* for 2 h. The pellet was suspended in 10 vols. 20 mM sodium acetate using a Dounce homogenizer and centrifuged at 175 000 × *g* for 1 h. This latter pellet was suspended at 5 mg protein/ml in 10 mM sodium acetate (pH 6.5) and termed the crude particulate fraction.

Enzyme assays. The standard assay (0.5 ml) for α-D-mannosidase contained 0.4 mM *p*-nitrophenyl-α-D-mannopyranoside (Sigma), 40 mM sodium acetate (pH 6.5) and was initiated by the addition of enzyme. After 15 min incubation at 25°C, the reaction was stopped by the addition of 0.2 ml 10% trichloroacetic acid (w/v). The sample was then centrifuged at 5900 × *g* for 10 min in a SS-34 rotor and the supernatant solution was added to 0.5 ml 1 M glycine, pH 10.4 [11]. The absorbance was determined at 400 nm and the amount of *p*-nitrophenol released during the reaction was calculated utilizing an extinction coefficient of $1.9 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$. Both a protein and substrate control were used. This reaction was linear with respect to time (0—40 min) and enzyme concentration (0.15—3.0 units of enzyme). Activity is expressed in μmol of *p*-nitrophenyl-α-D-mannopyranoside hydrolyzed per min. Specific activity is expressed as units per mg protein.

Aminopeptidase I was assayed as described by Masuda et al. [12] with minor modification. The assay mixture (0.5 ml) contained 1 mM leucyl-*p*-nitroanilide and 100 mM Tris · HCl (pH 7.6). After 30 min, the reaction was stopped by the addition of trichloroacetic acid and processed as described for the α-mannosidase assay. An extinction coefficient of $9.9 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$ at 400 nm was used to calculate the amount of *p*-nitroaniline released. Specific activity is expressed as μmol of leucyl-*p*-nitroanilide hydrolyzed per min per mg of protein.

Glucose-6-phosphate dehydrogenase was assayed as described by Gancedo and Gancedo [13] and phospho*enol*pyruvate carboxykinase was assayed as described by Gancedo and Schwerzmann [14]. Specific activity is expressed $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. The protein concentration was determined by the method of Lowry et al. [15] using dessicated bovine serum albumin as standard.

Solubilization procedures. In an attempt to solubilize α-mannosidase activity, 10—50 mg protein from the particulate fraction were suspended in 10 ml 20 mM sodium acetate (pH 6.5) containing either 1 M NaCl, 0.5% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1% (w/v) sodium cholate, or 1% (w/v) sodium *N*-lauroyl sarcosinate, by homogenization with a Dounce homo-

genizer. The sample was then centrifuged at 175 000 × *g* for 2 h at 3°C and the activity recovered in the supernatant solution was measured.

Results

Attempts to solubilize α-mannosidase. When SK-1 cells, exponentially grown on glucose or acetate, were broken by glass beads and centrifuged for 2 h at 175 000 × *g*, 80—85% of the activity was recovered in the pellet. When the supernatant solution from this centrifugation was centrifuged at 175 000 × *g* for another 2 h, 50% of the remaining activity was found in the new pellet. At no time was any soluble activity found that resembled the α-mannosidase activity reported by Kaya et al. [6], in that no activity was identified that exhibited a K_m greater than 100 μM with respect to *p*-nitrophenyl-α-mannopyranoside.

Tests on the solubilization of α-mannosidase activity suggested that the enzyme was not bound to the particulate fraction by ionic interactions, since 1 M NaCl released less than 5% of the activity. None of the detergents examined were able to solubilize more than 5% of the activity. When detergents, either alone or in combinations, were utilized sequentially on the same pellet in an attempt to solubilize the enzyme, 10% of the total activity and 80% of the total protein could be solubilized. Similar results were obtained with extracts prepared from cells grown in media containing either acetate or glucose as the carbon source.

Kinetic analysis. A crude particulate fraction prepared from SK-1 cells grown on rich acetate media or SK-1 cells harvested 6 h into sporulation were used to determine the kinetic parameters of this enzyme. The K_m of the enzyme for *p*-nitrophenyl-α-D-mannopyranoside was approx. 0.050 mM, independent of the presence of 0.1% (w/v) or 1% (w/v) Triton X-100. D-Mannose was found to be a linear competitive inhibitor of the enzyme with K_i 20 mM (*p*-nitrophenyl-α-D-mannopyranoside as substrate).

The pH profile of this enzyme activity was found to be very broad, exhibiting a pH optimum at approx. 6.3 (Fig. 1). There was no effect of 1% Triton X-100 on the pH optimum and none of the buffers used was found to be an inhibitor of this enzyme.

A series of cations, which are known to affect other α-D-mannosidases, were examined, including Zn^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, and Fe^{3+} [16—18]. These ions were tested at 0.1 and 1 mM concentrations and found to be without effect on this yeast α-D-mannosidase. The cation chelating agent EDTA (1 mM) was also without effect on this enzyme.

The sulfhydryl reagents, 2-mercaptoethanol, dithiothreitol, and dithioerythritol, were examined at 1 mM and found not to affect enzyme activity. *p*-Chloromercuriphenyl sulfonic acid, however, strongly inhibited the enzyme, causing a 50% decrease in enzyme activity at 0.35 mM (when treated with inhibitor for 2 min in the absence of substrate). The substrate, *p*-nitrophenyl-α-D-mannopyranoside, prevented this inhibition. When the enzyme was preincubated for 2 min with 1 mM *p*-chloromercuriphenyl sulfonic acid, the enzyme was totally inactivated. The addition of 10 mM 2-mercaptoethanol or 10 mM dithiothreitol after preincubation (which reacts with and inactivates the

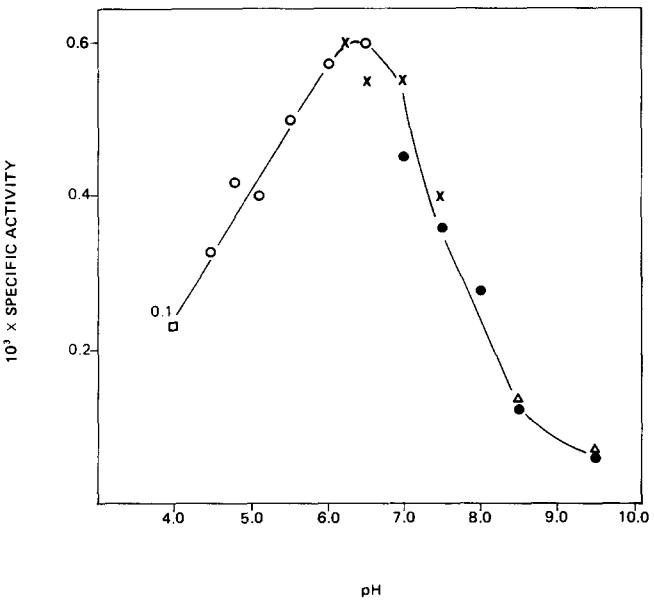

Fig. 1. Effect of pH on α-mannosidase activity. Crude particulate fraction from acetate grown cells ($6 \cdot 10^{-4}$ units/mg) was incubated with 0.4 mM *p*-nitrophenyl-α-D-mannopyranoside and either 100 mM sodium citrate (□), sodium acetate (○), potassium phosphate (X), Tris · HCl (●) or glycylglycine buffer (△), in a final volume of 0.5 ml for 15 min at 25°C and assayed as described in Methods.

TABLE I

EFFECT OF SUBSTRATE ON *p*-CHLOROMERCURIPHENYL SULFONIC ACID INHIBITION OF α-D-MANNOSIDASE

Crude particulate fraction (1 mg; $5 \cdot 10^{-4}$ units/mg) from acetate grown cells were incubated in the presence of 4 mM sodium acetate (pH 6.5) and 2 mM *p*-chloromercuriphenyl sulfonic acid, 10 mM 2-mercaptoethanol, or 0—4 mM *p*-nitrophenyl-α-D-mannopyranoside, adjusted with water to 0.5 ml. After 2 min at 25°C, all samples were adjusted to a final concentration of 10 mM 2-mercaptoethanol, 4 mM *p*-nitrophenyl-α-D-mannopyranoside, and 2 mM *p*-chloromercuriphenyl sulfonic acid (final addition) in 1.0 ml. This mixture was incubated for 30 min at 25°C and the release of *p*-nitrophenol was measured as described in Materials and Methods. The assay was initiated by the addition of enzyme to the well-mixed sample. Enzyme blanks (stopped by the addition of trichloroacetic acid immediately after preincubation) were used as zero-time controls.

Sample	Preincubation mixture			
	2-Mercaptoethanol (10 mM)	Substrate (mM)	Inhibitor * (2 mM)	Specific activity $1 \cdot 10^3$ units/mg
1	—	0	—	0.45
2	+	0	—	0.45
3	—	4	—	0.45
4	—	0	+	0.01
5	+	0	+	0.4
6	0	4	+	0.4
7	0	2	+	0.38
8	0	1	+	0.21
9	0	0.1	+	0.17
10	0	0.1	+	0.06

* The inhibitor is *p*-chloromercuriphenyl sulfonic acid.

remaining *p*-chloromercuriphenyl sulfonic acid), had no effect on the inhibition. When the preincubation with *p*-chloromercuriphenyl sulfonic acid was performed in the presence of 4 mM *p*-nitrophenyl-α-D-mannopyranoside, only a slight inhibition of activity is observed. This blockage of *p*-chloromercuriphenyl sulfonic acid inhibition was dependent on the concentration of substrate utilized (Table I). Preincubation with 2-mercaptoethanol or 4 mM *p*-nitrophenylα-D-mannopyranoside in the absence of *p*-chloromercuriphenyl sulfonic acid had no effect on enzyme activity. The specificity required for blocking *p*-chloromercuriphenyl sulfonic acid inhibition was shown by the fact that 4 mM *p*-nitrophenyl-α-D-glucopyranoside did not provide any protection to the enzyme when preincubated in the presence of *p*-chloromercuriphenyl sulfonic acid (unpublished data). These data suggest that the *p*-chloromercuriphenyl sulfonic acid sensitive sulfhydryl group is located within the substrate binding site of the enzyme, and hence is protected by the substrate.

The particulate preparation of acetate grown cells, washed with 1% (w/v) Triton X-100, did not give any detectable hydrolysis (less than 1 nmol of *p*-nitrophenol released) of the *p*-nitrophenyl derivatives of β-D-glucose, α-D-galactose, β-D-galactose, α-L-fucose, β-D-fucose or β-D-glucuronide, when incubated at 4 mM under assay conditions identical to α-mannosidase. The amount of extract used in these assays catalyzed the hydrolysis of 50 nmol of *p*-nitrophenyl-α-D-mannopyranoside in 30 min under standard assay conditions.

Modulation of α-D-mannosidase levels in cells. In an attempt to examine how the cell regulated the level of α-D-mannosidase, cells were grown on rich media containing either glucose, mannose, galactose, pyruvate, acetate, or lactate, and minimal media containing glucose or galactose as the carbon or energy source. After growing in these media for at least 6 generations, the cells were assayed for α-mannosidase and found to have a specific activity of 0.03, 0.07, 0.06, 0.19, 0.22, 0.27, 0.06, and 0.14 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein, respectively. When the specific activity of α-D-mannosidase was plotted against the doubling time of various cultures it was found that the specific activity of the enzyme was inversely proportional to the doubling time, increasing as the growth rate of the culture decreases. These results suggest that the enzyme is under catabolite repression.

In order to test whether α-D-mannosidase is regulated by catabolite inactivation [19], 2% glucose was added to a culture growing exponentially on glycerol. As expected (Fig. 2), the specific activity of the enzyme decreased until it attained the enzyme level characteristic of glucose growth. The kinetics of this loss of enzyme activity indicate that no catabolite inactivation occurred in this system. Phospho*enol*pyruvate carboxykinase, which was utilized as a control, gave similar results although it has been reported that this enzyme is rapidly inactivated after addition of glucose in other strains of baker's yeast [14]. Glucose-6-phosphate dehydrogenase, as expected, showed only a minor change in activity during this shift of carbon sources.

Since it is apparent that the enzyme was at a high level during periods of slow growth (when rapid metabolic changes may be expected to occur), it was of interest to examine this enzyme during the process of sporulation. Cells were grown on acetate presporulation media and sporulated in 1% potassium acetate media. It can be seen that the enzyme rapidly increased (approx. 8-fold) in

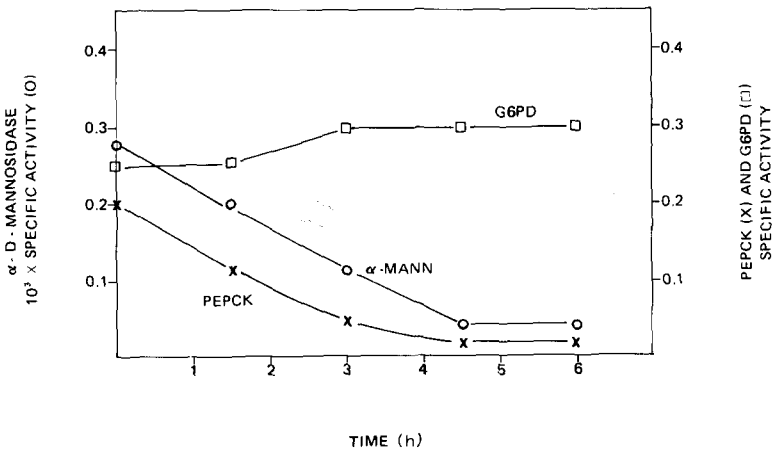

Fig. 2. Loss of α-mannosidase (α-MANN) activity after glucose addition to cells growing on glycerol. SK-1 cells were grown on a rich 2% (w/v) glycerol medium until the cell concentration was $4 \cdot 10^6$ cells/ml. Glucose (2%, w/v) was then added to these cells and the cells were sampled at 1.5-h intervals for α-mannosidase (○), phospho*enol*pyruvate carboxykinase (X), and glucose-6-phosphate dehydrogenase (G6PD) (□). When glucose was added to the culture it immediately started exponential growth with a doubling time of 90 min and grew at that rate for the remainder of the experiment.

strains that sporulate, such as SK-1 or AP-1-a/α (Fig. 3). However, strain AP-1-α/α, (which cannot sporulate) showed only a 3.4-fold increase in enzyme activity. This increase in activity occurred almost entirely within the first 7 h in sporulation media, in comparison to AP-1-a/α, which increases in specific activity for 12 h, until the 4-spored asci are formed. When strain α-131-20 (which also cannot sporulate) was incubated in sporulation media, results similar to those obtained for AP-1-α/α were obtained, with an increase in specific activity

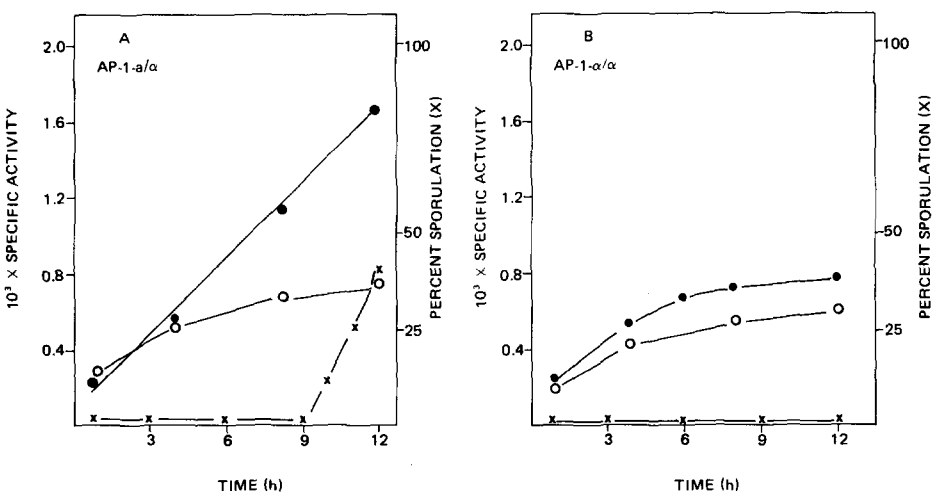

Fig. 3. Alteration of α-mannosidase activities during incubation in sporulation media. AP-1-a/α (A) or AP-1-α/α (B), pregrown in rich acetate medium and incubated in sporulation medium as described in Materials and Methods were sampled at various times during sporulation and assayed for α-mannosidase (●), aminopeptidase I (○), and the percentage of ascospores (X) as described in Materials and Methods.

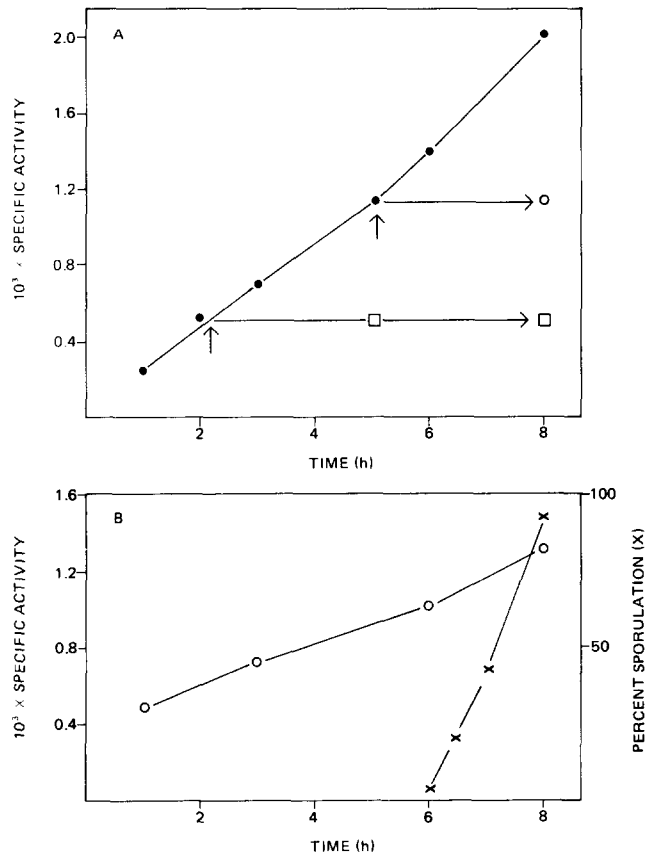

Fig. 4. Alteration in α-mannosidase during sporulation of SK-1 and the effect of cycloheximide on enzyme levels. SK-1 was pregrown and sporulated as described in Materials and Methods. In (A) the change in specific activity in α-mannosidase during sporulation (•) was measured as described in Materials and Methods. In experiments performed simultaneously, 100 μg/ml of cycloheximide were added at 2.1 h (□) or 5 h (○) and assayed for α-mannosidase activity after a total of 8 h of incubation in sporulation media. In (B) the activity of aminopeptidase I (○) and the percentage of ascospores (X), assayed as described in Materials and Methods, is presented throughout sporulation in strain SK-1.

occurring only during the first 6 h of incubation (unpublished data). When crude extracts of sporulating SK-1 cells (T_6) were centrifuged at 175 000 × *g* for 1 h, 85% of the activity was located in the pellet. Glucose-6-phosphate dehydrogenase (which like aminopeptidase I was used as a control) remained at the same concentration during sporulation (unpublished data). Aminopeptidase I increased 1.5—2-fold in all strains, independent of the cell's sporulation ability.

In order to examine whether the increase in α-mannosidase activity during sporulation in SK-1 cells was due to new protein synthesis or the activation of a precursor enzyme, cycloheximide was added to sporulating cells at 2 and 5 h after incubation in sporulation medium. This protein synthesis inhibitor [20] stopped the synthesis of α-mannosidase immediately (Fig. 4), indicating that continual protein synthesis was required for the increase in enzyme activity.

Discussion

The α-mannosidase described appears to have a number of unique properties not found in other systems. The yeast α-D-mannosidase was unlike any of the three rat liver enzymes [16—18] in that it had a low K_m with respect to *p*-nitrophenyl-α-D-mannopyranoside, did not require cations for activity, and was not affected by EDTA, as was the rat liver lysosomal enzyme (18, 21). Although the yeast enzyme is probably membrane bound and is inhibited by *p*-chloromercurophenyl sulfonic acid as is the rat liver Golgi enzyme [16], it is not solubilized by detergents and is not activated by Co^{2+} or Zn^{2+}.

This yeast α-D-mannosidase was also different from that reported by Kaya et al. [6] from baker's yeast. Not only was this enzyme tightly bound to the particulate fraction of the cell, but it has a K_m with respect to *p*-nitrophenyl-α-D-mannopyranoside which is 20-fold lower than the K_m reported by Kaya et al. No enzyme was identified which has a K_m similar to that reported by Kaya et al. in the strains of yeast used in this study. These facts suggest that this is a different and distinct enzyme.

The level of enzyme was modulated over a 50-fold range of concentration. The enzyme is apparently controlled by catabolite repression, since the enzyme concentration varies inversely with the growth rate. The concentration of α-D-mannosidase, in cells growing on mannose as carbon source, is the same as would be predicted assuming that catabolite repression is the major factor controlling enzyme synthesis. Mannose, therefore, does not function as an end product inhibitor of enzyme synthesis. When enzyme synthesis is blocked by the addition of glucose, the specific activity decreases in a manner which would be expected to occur if the enzyme was diluted out by cell division until a new steady state level of enzyme is reached. The experiments presented do not, however, rule out an increased degradation rate with an unchanged synthesis rate.

The enzyme concentration apparently increases 8-fold during sporulation. Although the concentration also increases in strains of yeast such as AP-1-α/α which do not sporulate, the increase is less than one-half as large as sporulating strains and is most likely a consequence of the step-down conditions obtained when cells are incubated in sporulation medium. Also, it should be noted that the increase of α-D-mannosidase activity levels off in about 7 h of incubation in non-sporulating strains of yeast (a time at which normal sporulation is one-half completed) but keeps increasing in sporulating strains until ascospores are completely formed. The late increase in enzyme which occurs after 7 h in sporulation medium is specific to sporulation, suggesting that α-D-mannosidase may have an important role late in the sporulation process.

Acknowledgements

These studies were supported in part by grant A-683 from the Robert A. Welch Foundation and the College of Science Organized Research Fund at Texas A & M University.

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