Biochimica et Biophysica Acta, 391 (1975) 67—74

BBA 67490

ACID PHOSPHATASE AND ADENOSINE TRIPHOSPHATASE ACTIVITIES IN THE CELL WALL OF BAKER'S YEAST

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(Received October 14th, 1974)

Summary

In order to establish whether a specific adenosine triphosphatase is present in yeast cell wall, hydrolysis rates for *p*-nitrophenylphosphate (acid phosphatase activity) and for ATP (ATPase activity) were compared under various conditions. Rate determinations were made with both, intact cells and with preparations containing secreted enzymes from protoplasts. Acid phosphatase and ATPase activities had the same pH profile and were susceptible in the same way to the repression by orthophosphate and to the inhibition by 2-deoxyglucose.

The Lineweaver-Burk plot shows biphasic kinetic behaviour for the hydrolysis of either *p*-nitrophenylphosphate or ATP. This suggests the existence of two enzymes with different affinities for the substrates, or one enzyme with at least two active sites. The two activities differ in thermostability and only one activity could be completely abolished by heat treatment. The thermostable enzyme activity had K_m values of 0.475 mM for *p*-nitrophenylphosphate, and 0.040 mM for ATP.

ATP behaved as a partially competitive inhibitor of *p*-nitrophenylphosphate hydrolysis. Substrate competition studies showed that only a non-specific acid phosphatase is responsible for the hydrolysis of ATP.

Introduction

It is now well established that the cell wall of *Saccharomyces cerevisiae* contains an acid phosphatase (orthophosphoric monoester phosphohydrolase (acid optimum) EC 3.1.3.2) with a pH optimum 3—4 [1—4].

Protoplasts from baker's yeast release acid phosphatase into the medium, and the secreted enzyme appears to be identical with that located in the wall of intact yeast cells [5].

Abbreviation: NPh-*P*, *p*-nitrophenylphosphate.

Several authors have suggested that, in addition to acid phosphatase, the cell wall of *S. cerevisiae* contains a specific ATPase, optimally active at pH 3.5 [1,6,7,8]. On the other hand, Ohwaki and Lewis [9] after investigating the ATPase activity in the cell wall of *Saccharomyces carlsbergensis*, attributed the ATP hydrolysis entirely to the acid phosphatase.

For a decision on this matter more detailed data are required, and we have carried out a series of experiments to obtain such data.

Materials and Methods

Materials

The yeast species used in the experiments was *S. cerevisiae* (strain 367, Technological Faculty Collection, Zagreb). The yeast was cultured aerobically, in phosphate-poor medium containing 5% glucose, 0.1% $(NH_4)_2SO_4$ and 0.5% phosphate-free yeast extract [10]. Required quantities of 2-deoxyglucose or inorganic phosphate were added to this medium as stated. During cultivation (14 h) the yeast was shaken in 100-ml batches, at 28° C.

Protoplasts were prepared from yeast cells by using *Helix pomatia* snail gut juice obtained from L'Industrie Biologique Française (Genevilliers, France) [5,11]. The protoplasts were incubated in Markham-mannitol medium [5].

All chemicals used were analytical grade.

The enzyme assay was carried out either with a 1% suspension of intact cells, or with a dialysed enzyme preparation obtained by secretion from protoplasts [5]. After dialysis the enzyme was concentrated by ultrafiltration under N_2 and stored in 0.2 M sodium acetate buffer, pH 3.8, at +4° C.

Analytical methods

Acid phosphatase activity was determined with *p*-nitrophenylphosphate (NPh-*P*) as the substrate [12]. One unit of enzyme activity is equivalent with the formation of 1 μmol of *p*-nitrophenol per min under specified conditions (0.2 M sodium acetate buffer, pH 3.8, 30° C). Specific activities are expressed in units/mg of dry matter (when intact cells were used) or in units/ml of the enzyme preparation secreted from protoplasts.

The enzymatic hydrolysis of ATP was carried out in 0.2 M acetate buffer, pH 3.8, at 30° C. Aliquots were taken within the first 2—3 min only, to minimise the influence of product (ADP) hydrolysis, and the reaction was stopped by mixing with 1 M $HClO_4$. P_i liberated by ATP hydrolysis was determined by the method of Marsh [13]. The unit of ATPase activity was expressed as μmol of P_i released from ATP per min. The values were corrected for non-enzymatic hydrolysis of ATP and for endogenous P_i.

Results and Discussion

Acid phosphatase and ATPase activities during cultivation of yeast

Acid phosphatase and ATPase activities of the intact cells were determined, at intervals, during the yeast growth. Maximal increase of acid phosphatase activity was reached between 8 and 10 h of cultivation and maximal

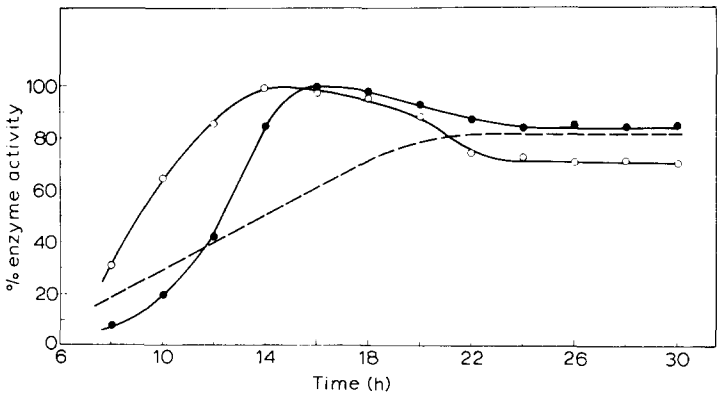

Fig. 1. Acid phosphatase and ATPase activities expressed in percent of the maximal activity measured in the yeast cells during cultivation in a phosphate-poor medium. ○———○, acid phosphatase activity; ●———●, ATPase activity. The growth is presented by a dashed line.

increase of ATPase activity was between 12 and 14 h (Fig. 1). Thus maximum rates of hydrolysis of NPh-*P* and ATP occur within the log phase of growth.

2-Deoxyglucose was reported to inhibit the biosynthesis of acid phosphatase [14]. Addition of 2-deoxyglucose (1 and 2 mg/ml) at the time of maximal increase of acid phosphatase and ATPase activities, caused a suppression of both activities to nearly the same extent (Fig. 2).

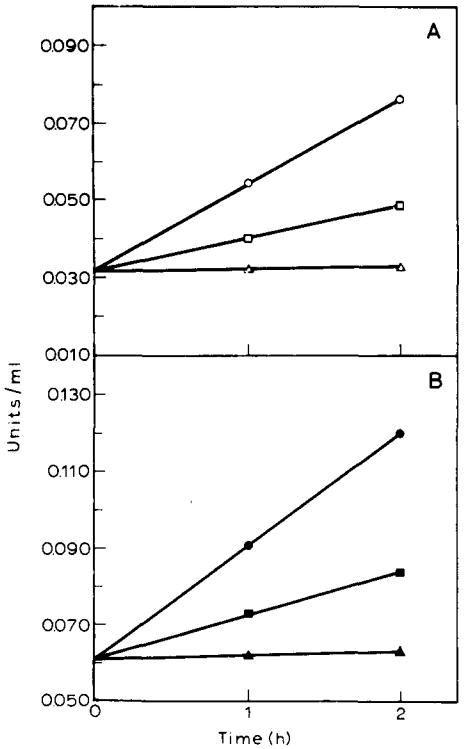

Fig. 2. Effect of 2-deoxyglucose on acid phosphatase and ATPase activities during the growth in a phosphate-poor medium. (A) Acid phosphatase activities. 2-Deoxyglucose was added to the medium after 8 h of growth. ○———○, zero; □———□, 1 mg/ml; △———△, 2 mg/ml. (B) ATPase activities. 2-Deoxyglucose was added to the medium after 12 h of growth. ●———●, zero; ■———■, 1 mg/ml; ▲———▲, 2 mg/ml.

Addition of inorganic phosphate to the growth medium is known to induce a marked repression of acid phosphatase [15]. We found that yeast cells grown in presence of 1 mM P_i in the medium, developed considerable less ATPase activity than controls cultured in absence of P_i.

The results described above demonstrate a close similarity in the behaviour of yeast acid phosphatase and ATPase activities.

Both activities had the pH optima within the same range.

Acid phosphatase and ATPase activities during secretion from protoplasts

Fig. 3 represents the time course of the secretion of enzymes from yeast protoplasts in Markham-mannitol medium. After 7 h of incubation the secretion stops, acid phosphatase and ATPase activities remaining constant for the rest of the observation period. Van Rijn et al. [5] observed a renewal of acid phosphatase secretion after supplying the medium with glucose. We found that, under the same conditions, ATPase secretion was resumed as well.

The ratio of ATPase activity and acid phosphatase activity, determined in the enzyme preparation secreted by protoplasts, was the same as the activity ratio of intact yeast cells. This means that ATPase activity was secreted to the same extent as acid phosphatase activity.

Thermal stability

Thermal stability of the preparation secreted by protoplasts with NPh-*P* and ATP as substrates, is shown in Fig. 4. At the two temperatures, 43 and 50° C, the hydrolysis of either substrate is retarded by similar dicrements. However, the relationship between log % activity and time is obviously non-linear. The curve obtained for both substrates might be interpreted as suggesting the existence of two independent enzyme systems, or one enzyme with, at least, two binding sites having different thermostability.

Effect of substrate concentration

The Lineweaver-Burk plots for NPh-*P* and ATP as substrates, are shown in Figs 5 and 6, respectively. Biphasic curves were obtained with either susbtrate

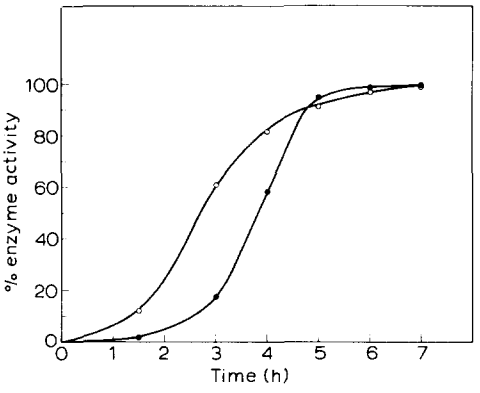

Fig. 3. Acid phosphatase and ATPase activities expressed in percent of the maximal activity reached during secretion from protoplasts. The protoplasts were incubated in Markham-mannitol medium. After centrifugation the supernatant was used for enzyme assays. For legends, see Fig. 1.

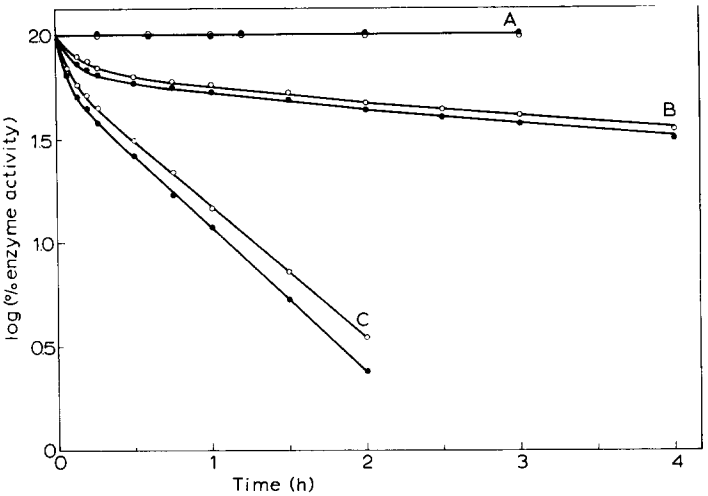

Fig. 4. Thermal stability of the secreted enzyme. The enzyme solution was incubated for various time intervals: A, at 40 °C; B, at 43 °C; C, at 50 °C. The remained activity was measured at 30 °C. For legends, see Fig. 1.

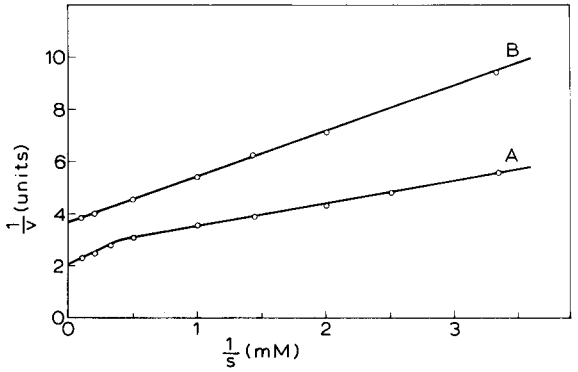

Fig. 5. Dependence of initial velocity on *p*-nitrophenylphosphate concentrations. A, enzyme preparation secreted by protoplasts; B, the same preparation previously incubated for 2 h at 43 °C.

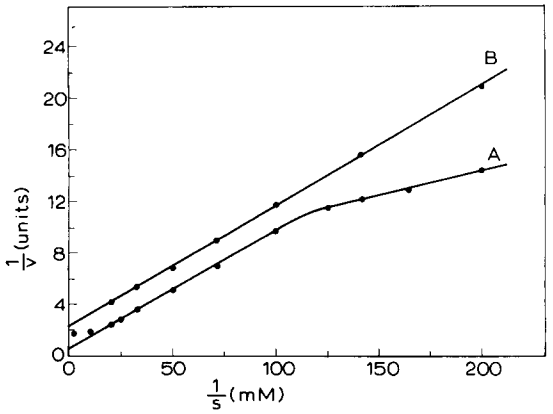

Fig. 6. Dependence of initial velocity on the concentration of ATP. A, enzyme preparation secreted by protoplasts; B, the same preparation preincubated at 43 °C for 2 h.

(labelled A in both figures). The two linear parts in both instances had distinctly differing slopes [16], which supports the assumption of the existence of two enzymes, or one enzyme with multiple binding sites. Assuming the existence of two different enzymes, we might be able to explain the difference in ATPase and acid phosphatase activities observed during the first phase of these enzymes' secretion by the protoplasts (see Fig. 3). The same difference was observed during the yeast growth. It might be true that the biosynthesis of the two enzymes does not occur simultaneously, but the enzyme with the K_m value for NPh-*P* lower and that for ATP higher is synthesized first.

From the curves on double reciprocal plots two limiting K_m values could be graphically estimated. At NPh-*P* concentrations below 2 mM, extrapolation of the respective linear portion of the curve yielded an apparent K_m of about 0.32 mM. The apparent K_m at concentrations above 2 mM was about 1.20 mM. For ATP concentrations below 0.008 mM the apparent K_m value was approx. 0.06 mM and for concentrations higher than 0.008 mM the K_m value was about 0.135 mM.

When the enzyme preparation was kept at 43°C for 2 h, its low K_m ATP-hydrolysing and high K_m NPh-*P*-hydrolysing activities were completely abolished and straight lines were obtained in Lineweaver-Burk plots (Figs 5 and 6, curves B). From the curves labelled B on both figures the apparent K_m for NPh-*p* was 0.475 mM and that for ATP was 0.040 mM.

Substrate competition

If one of the substrates were a competitive inhibitor of the hydrolysis of the other, it would be possible to establish whether the substrates are hydrolyzed by one and the same, or by different enzymes. If both substrates are hydrolyzed by a single enzyme, the experimentally determined, and calculated (Eqn 1) rates should be equal.

For these experiments the thermally less stable activity was eliminated by heat treatment at 43°C.

ATP actually inhibited the hydrolysis of NPh-*P*. Results plotted in double reciprocal manner showed competitive inhibition within a range of ATP concentrations from 0.02 to 0.50 mM. Experimental points also lay along a straight line on a Dixon plot. At higher ATP concentrations, however, a hyperbolic Dixon plot was obtained, indicating a partially competitive inhibition.

In this type of inhibition the following equations are valid:

$E + S \rightleftharpoons ES \rightarrow E + P$	dissociation constant K_m
$E + I \rightleftharpoons EI$	dissociation constant K_i
$EI + S \rightleftharpoons EIS$	dissociation constant K_i'
$ES + I \rightleftharpoons EIS$	dissociation constant K_i''
$EIS \rightarrow EI + P$	

ES and EIS break down to products at the same rate.

In the experiments performed, for instance, with 10 mM NPh-*P* in the presence of 0.4 mM ATP the rate of hydrolysis of NPh-*P* was 0.179 unit (V = 0.270 unit, K_m = 0.475 mM, K_m for ATP = 0.040 mM). The reaction rate

calculated from the equation for fully competitive inhibition [17]

$$v = \frac{V}{1 + \frac{K_m}{S}\left(1 + \frac{I}{K_i}\right)} \quad (1)$$

was 0.177 unit. The value of K_m for ATP was substituted for K_i [18]. At higher concentrations of ATP (10 mM ATP and 10 mM NPh-*p*), under the same conditions as above, the observed reaction rate was 0.042 unit. The calculated reaction rate (Eqn 1) was 0.0208 unit which represents the rate of breakdown of ES complex. This discrepancy is due to partially competitive inhibition becoming prominent at higher ATP concentrations. The reaction rate is the sum of rates for breakdown of ES and EIS complexes. Therefore, the difference between the reaction rate obtained experimentally (0.042 unit) and that calculated from Eqn 1 (0.0208 unit) represents the breakdown rate of complex EIS (0.021 unit). This rate is expressed by Eqn 2 [17]

$$v = \frac{V}{1 + \frac{K_i'}{S}} \quad (2)$$

As the value of v is known (0.021 unit) we are able to calculate constant K_i', determination of which is rather intricate. The calculated value was 117 mM. As we can see, the dissociation constant K_i' is rather high, which is the reason why the partially competitive inhibition could not be distinguished from the fully competitive type at low inhibitor concentrations.

By using the calculated value of K_i' we are now able to calculated the reaction rate from the rate equation for partially competitive inhibition (Eqn 3) [17]

$$v = \frac{V}{1 + \frac{K_m}{S}\left(\frac{1 + I/K_i}{1 + IK_m/K_iK_i'}\right)} \quad (3)$$

The calculated reaction rate was 0.039 unit, which is close enough to the experimental rate. This accordance between experiment and theory shows that a single enzyme takes part in the hydrolysis of both substrates and, according to our other results, it may be assumed that no specific ATPase is present in the yeast, either in the cell wall, or in the protoplast secretion.

In addition it might be suggested that the thermostable enzyme posseses two separate active sites, one for binding NPh-*p* and one for binding ATP. This assumption is plausible because the results are compatible with a partially competitive system in which inhibitor and substrate combine with different groups on the enzyme [19]. This opinion seems to be supported by the different action of phenylmercuric acetate on the hydrolysis of NPh-*p* and ATP [8].

Furthermore, the thermostability studies and the studies of the effect of substrate concentration, indicate that there are either two acid phosphatases, or one, with at least two active sites in the yeast cell wall. This alternative will be decided upon after fractionation and purification of the enzyme preparation secreted from protoplasts, which will be the subject of our further investigations.

Acknowledgment

This work was supported by a grant from Republic's Research Fund of Croatia.

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