Biochimica et Biophysica Acta, 657 (1981) 482—494

BBA 69196

A PARTICULATE FORM OF ALKALINE PHOSPHATASE IN THE YEAST, *SACCHAROMYCES CEREVISIAE*

JAMES K. MITCHELL, WILLIAM A. FONZI, JAMES WILKERSON and DENNIS J. OPHEIM *

Department of Biology, Texas A & M University, College Station, TX 77843 (U.S.A.)

(Received September 29th, 1980)

Key words: Alkaline phosphatase; Particulate enzyme, (S. cerevisiae)

Summary

A new form of alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) has been identified in the yeast *Saccharomyces cerevisiae*. Utilizing either synthetic or natural substrates, the enzyme exhibited a broad pH activity curve with maximum activity between 8.5 and 9.0. The enzyme was nonspecific with respect to substrate, attacking a variety of compounds containing phosphomonoester linkages, but has no detectable activity against polyphosphate, pyrophosphate or phosphodiester linkages. The enzyme exhibited an apparent K_m of 0.25 mM with respect to *p*-nitrophenyl phosphate, 0.38 mM with respect to α-naphthyl phosphate, and 1.0 mM with respect to 5'AMP. The enzyme is regulated in a constitutive manner and its activity does not increase during phosphate starvation or sporulation, as does the repressible alkaline phosphatase. The enzyme is tightly bound to a particulate fraction of the cell, tentatively identified as the tonoplast membrane. It is not solubilized by treatment with high concentrations of NaCl, KH_2PO_4 or chaotropic agents. Triton X-100 (0.1%) solubilizes 12% of the particulate activity. This enzyme is differentiated from the other alkaline phosphatases found in yeast by its chromatographic elution from DEAE-cellulose, kinetic parameters, heat stability and pH stability, as well as its particulate nature. This particulate alkaline phosphatase was found in every strain examined. It has a significantly lower specific activity in the *phoH* mutant and a higher activity in the acid phosphatase constitutive mutant A137.

Present address: Department of Medical Laboratory Sciences, Quinnipiac College, Hamden, CT 06518, U.S.A.

Introduction

A number of different alkaline phosphatases (orthophosphoric-monoester phosphohydrolase (alkaline optimum, EC 3.1.3.1) are found in the yeast *Saccharomyces cerevisiae* [1—3]. These enzymes are distinguished on the basis of their substrate specificity, elution from ion-exchange resins, regulation and genetic analysis. The best characterized alkaline phosphatase is a nonspecific enzyme with a pH optimum of 8.5—9.0 [3—5]. This enzyme is repressed by inorganic phosphate [3,5] and is absent in the *phoH* mutant [5]. A second alkaline phosphatase has been reported which is regulated in a constitutive manner and is relatively specific with respect to substrate, attacking *p*-nitrophenyl phosphate but no other substrate at a significant rate [3—5]. The natural substrate for this enzyme has not been identified. This constitutive alkaline phosphatase is not affected by the *phoH* mutation [5]. Both enzymes require a divalent cation for activity [3—5]. A third alkaline phosphatase has been identified which specifically attacks histidinyl phosphate [4]. All three enzymes can be separated by chromatography on DEAE-cellulose [4,5].

During the course of our studies on the repressible alkaline phosphatase, a previously unreported form of nonspecific alkaline phosphatase was identified. This form of alkaline phosphatase was differentiated from known phosphatases by a number of biochemical criteria. In this manuscript we report the distinguishing physical and kinetic properties of this phosphatase.

Materials and Methods

Yeast strains. Yeast were grown and transferred daily on agar slants composed of 1% yeast extract/1% proteose peptone/1% glucose or galactose. Strains α-131-20 and A364A were obtained from A.K. Hopper [6]. Strain SK-1, which is a homothallic prototroph capable of extensive and synchronous sporulation, was obtained from R. Roth [7]. Strain ts 136 (*rna 1—1)* and X-2180-1B were obtained from the Yeast Genetic Stock Center (Berkeley, CA). Strain AL-14-1D (*phoH*), which has been reported to be deficient in repressible alkaline phosphatase activity, was obtained from Yasuji Oshima [5]. Strain A 137 (*acp 1—2, pho 80*), a strain isolated as a constitutive high acid phosphatase producer [8], was obtained from the Yeast Genetic Stock Center (Berkeley, CA). LB60-5A, a glucosamine-requiring mutant, was obtained from Clinton Ballou [22].

Cultivation of the microorganism. Yeast cells were grown in a medium consisting of 1% yeast extract/1% proteose peptone/1% glucose (medium A) or 1% potassium acetate (medium B). 1 l medium, contained in a 2-l Erlenmeyer flask, was inoculated with cells from a yeast extract/proteose peptone/acetate slant and incubated for 12—16 h at 30° C, on a orbital shaker (300 rev./min, 1.5 inch orbit). Glucosamine (1 mg/ml) was added for the growth of LB-60-5A. Cells grown in medium A were derepressed for alkaline phosphatase by resuspending 1% NaCl-washed cells in 1% glucose at a cell density of $2 \cdot 10^7$ cells/ml and incubating the cells as described above.

Yeast cells were grown and sporulated by a modification of the procedures described by Roth and Halvorson [9]. Cells were grown as described above in

medium B to a density of $2 \cdot 10^7$—$4 \cdot 10^7$ cells/ml. The culture was centrifuged at 4000 rev./min ($3240 \times g$) in a Sorvall GS-3 rotor for approx. 1 min at 4°C. The pellet was washed twice with 20 ml sterile 0.85% NaCl and suspended in 500 ml sporulation medium (1% potassium acetate) at a density of $3 \cdot 10^7$ cells/ml, in a 2 l flask, and shaken at 30°C as previously described [10].

Assay of enzymes. Alkaline phosphatase activity was assayed by measuring the hydrolysis of synthetic phosphomonoester substrates which produce either fluorescent or light absorbent products, or by measuring the release of inorganic phosphate. In all cases, the reaction mixture (1 ml) contained 250 mM Tris-HCl, pH 8.5/10 mM $MgSO_4$/10 μM $ZnSO_4$/substrate/enzyme. The synthetic substrates *p*-nitrophenyl phosphate (dicyclohexylammonium salt), α-naphthyl phosphate (disodium salt) and 4-methylumbelliferyl phosphate were used at a concentration of 1, 5.0 and 1 mM, respectively. After incubation with enzyme at 30°C for 5—15 min, the reaction was stopped by adding 1 ml 2 M glycine, pH 11.0. The *p*-nitrophenyl released was measured spectrophotometrically at a wavelength of 400 nm; the α-naphthol and 4-methylumbelliferone released were quantitated by measuring their fluorescence at 450 nm or 425 nm, respectively, when excited at 345 nm. The inorganic phosphate released was measured as described by Fiske and SubbaRow [25] or Ohnishi et al. [26]. The reaction was linear with respect to time and enzyme concentrations, for each substrate over the time intervals and range of concentrations utilized. Substrate and enzyme blanks were run for each assay. In each case, 1 unit of activity is defined as the production of 1 μmol product/min.

α-Mannosidase was measured as described by Opheim [10], glucose-6-phosphate dehydrogenase by the procedure of Gancedo and Gancedo [11], succinate dehydrogenase as described by Racker [12], and protease B as described by Juni and Heym [13]. Protein was determined by the procedure of Lowry et al. [14].

Preparations of extracts. The preparations of cell extracts for enzyme assay was initiated by the addition of approx. 500 g ice to a 1 culture. The cells were collected by centrifugation at 5000 rev./min ($5140 \times g$) in a Sorvall GS-3 rotor for 5 min at 4°C. The cell pellet was washed once with 20 ml cold 0.85% NaCl solution. Approx. 3 g cells were suspended in 7 ml buffer containing 100 mM imidazole (pH 7.0)/10 mM $MgSO_4$/10 μM $ZnSO_4$/1 mM phenylmethylsulfonyl fluroride. The cell suspension was mixed with 8 g 0.3 mm glass beads and the cells were broken in a Braun homogenizer cooled to approx. 4°C with liquid CO_2. At least 90% of the cells were broken by this procedure. The homogenate was centrifuged at 4000 rev./min for 5 min in a Sorval SS-34 head to remove glass beads and unbroken cells. The supernantant solution, designated as the crude extract, was assayed for alkaline phosphatase activity. The particulate form of the enzyme was separated from the soluble enzyme forms by centrifugation for 1 h at $200\,000 \times g$ in a Beckman Ti-60 rotor. The pellet was resuspended with a Dounce homogenizer in 15 ml 100 mM imidazole (pH 7.0)/10 mM $MgSO_4$/10 μM $ZnSO_4$. Specific activity of the particulate enzyme is expressed as μmol phosphate released/min per mg protein.

Repressible alkaline phosphatase purification. The repressible alkaline phosphatase was purified to a specific activity of 360 μmol *p*-nitrophenyl phosphate hydrolyzed/min per mg protein by a procedure involving DEAE-cellulose

chromatography followed by antibody chromatography (Fonzi, W. Opheim D., unpublished data).

Electrophoresis and localization of activity. Electrophoresis was performed in 10 cm 7% polyacrylamide gels at pH 8.9, as described by Laemmli [15], except that there was no sodium dodecyl sulfate in this sytem. After electrophoresis at 3 mA/tube at 4°C for approx. 2 h, the bands of alkaline phosphatase activity were visualized by three different assay procedures. In one system, the gels were placed in capped test tubes containing 1 mM *p*-nitrophenyl phosphate/10 mM $MgSO_4$/100 mM Tris-HCl (pH 8.5) and gently shaken at 30°C. After 2—10 min, yellow bands appeared where *p*-nitrophenol was being released by the action of phosphatase. In the second system, 1 mM 4-methylumbelliferone phosphate was mixed with the pH 8.5 buffer, as described previously. After 5—15 min the Tris-HCl buffer solution was removed and 0.5 M potassium phosphate (pH 11) was added to the gel. When examined with a long wave ultraviolet light source, light blue bands appeared at the site where 4-methylumbelliferone was being produced. In the third system, the gel was gently shaken in 10 ml buffer containing 2.5 mM α-naphthyl phosphate/10 mM $MgSO_4$/100 mM Tris-HCl (pH 8.5) at 30°C. The enzyme activity could be localized in two ways. The gels could be suspended in 0.5 M potassium phosphate, pH 11, and examined with a long wave ultraviolet light, under which conditions the α-naphthol formed by the action of the enzyme fluoresces green. In the second method, 0.6 ml *N,N*-dimethyl formamide containing 10 mg tetrazolized *o*-dianisidine was added to the reaction mixture. In this system, the α-naphthol formed by the action of phosphatase reacts with the dye to form an insoluble black dye complex at the site of enzyme activity.

Solubilization of particulate phosphatase. The pellet formed by centrifuging the crude extract for 1 h at 200 000 × *g* was suspended in 15 ml 100 mM imidazole (pH 7.0)/10 mM $MgSO_4$/10 μM $ZnSO_4$/either 0.1 or 1.0% Triton X-100/1 M NaBr/1 M NaCl/1 M urea, or 100 mM potassium phosphate and homogenized with a Dounce homogenizer. The samples were centrifuged at 200 000 × *g* for 1 h and the activity recovered in the supernatant solution and pellet was measured.

Heat inactivation. Enzyme samples were equilibrated with 50 mM imidazole (pH 7.0)/10 mM $MgSO_4$ by passing them through a 20 cm P-10 (Pharmacia) column equilibrated with the same buffer. Samples (0.5 ml) of enzyme containing approx. 0.63 units of alkaline α-naphthyl phosphatase activity/ml were incubated at 45°C for various lengths of time up to 120 min. Samples were asaayed at 30°C for alkaline phosphatase activity. The percent of initial activity remaining after heat treatment was calculated.

pH Stability. Enzyme extract (30 μl) suspended in 25 mM Tris-HCl buffer (pH 8.5)/1 mM $MgSO_4$ was added to 1 ml 50 mM Tris acetate (pH 4—8) containing either 2 mM $MgSO_4$, 10 μM $ZnSO_4$, 0.5 mM $MnCl_2$ or 1 mM EDTA, and incubated at 4°C for 8 h. The pH of individual samples was confirmed to ensure that addition of extract did not alter the pH. A sample (10 μl) of the test solution was assyed in 1.0 ml reaction mixture containing 5 mM α-naphthyl phosphate/250 mM Tris-HCl (pH 8.5)/10 mM $MgSO_4$. The reaction was stopped with 1 ml 2 M glycine, pH 11, and measured as previously described.

Vacuole preparation. Spheroplasts were prepared as described by Schwemke

et al. [16]. Vacuoles were purified from spheroplasts by a variation of the procedure of Weimken et al. [17], using their floatation on Ficoll gradients. The purification was monitored by following the vacuolar enzyme markers protease B and α-mannosidase, the cytosolic marker glucose-6-phosphate dehydrogenase and the mitochondrial marker succinate dehydrogenase [18]. The vacuoles were lysed by the addition of 0.1% Triton X-100 and the tonoplast membrane was separated from soluble components by centrifugation at 200 000 × *g* for 1 h.

Results

Electrophoresis

Three forms of alkaline phosphatase activity could be demonstrated on polyacrylamide gels after electrophoresis of crude extracts prepared from derepressed cells. Fig. 1A displays the results obtained upon electrophoresis of crude extracts prepared from strain α-131-20, derepressed for 8 h in phosphate-less medium. The three forms of phosphatase were distinguished by both their position on the gel, as well as their differential activity toward the substrates *p*-nitrophenyl phosphate, α-naphthyl phosphate and 4-methylumbelliferyl phosphate.

The activity (Band I) at the top of the gel (cathode end) was active against all three substrates. The middle band (Band II) attacks 4-methylumbelliferyl phosphate and *p*-nitrophenyl phosphate, but not α-naphthyl phosphate. The most rapidly moving activity (Band III) attacks all three substrates. Strains SK-1, X-2180-1B, and LB60-5A give similar results. When crude extracts of α-131-20 cells grown in YPD medium supplemented with 20 mM potassium phosphate were electrophoresed the results in Fig. 1B were observed. Bands I and II were present at their normal intensities, but activity Band III was significantly reduced. If the assay was allowed to proceed for 5-times the

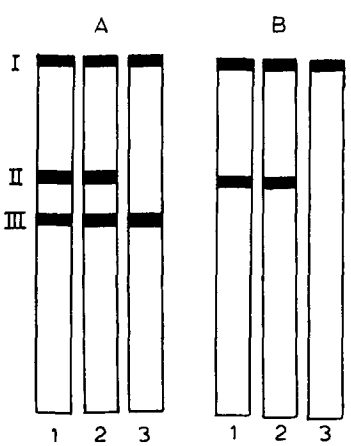

Fig. 1. Electrophoretic identification of mutiple alkaline phosphatases. Crude extracts of α-131-20 cells grown in low (A) or high phosphate (B) media were electrophoresed and assayed for alkaline phosphatase activity with (1) *p*-nitrophenyl phosphate, (2) methylumbelliferyl phosphate, and (3) α-naphthyl phosphate as substrate.

normal time, Band III activity appeared. When extracts of strains SK-1, X-2180-1B and LB60-5A were examined after growth in high phosphate media, activity Band III always had diminished activity, although there were considerable quantitative differences between strains.

Activity Band II apparently corresponds to the *p*-nitrophenyl phosphate specific, constitutive enzyme reported by Gorman and Hu [4] and later by Toh-e et al. [5]. Our data indicate that 4-methylumbelliferyl phosphate is also hydrolyzed by this enzyme (Fig. 1A). As has been previously reported [5], this enzyme does not attack α-naphthyl phosphate. Band III, which corresponds to the soluble repressible enzyme, attacks all three substrates. This activity is repressed by inorganic phosphate, has a broad substrate specificity and has the same elution pattern from DEAE-cellulose (Fonzi and Opheim, unpublished data), as that previously reported for the repressible alkaline phosphatase [3—5]. To the best of our knowledge, the top band of activity has not been previously reported in *S. cerevisiae*. All of these bands had significantly decreased activity when assayed in imidazole buffer, pH 7.0, indicating that they do not represent an acid phosphatase activity, Since Bands I and III had similar substrate specificities, these two forms were further analyzed for other similarities. Band II phosphatase activity was clearly different from the other enzymes, since it exhibits no detectable activity against α-naphthyl phosphate. The use of this substrate provides a convenient means of eliminating interference from Band II phosphatase activity.

Particulate nature of Band I

When a crude extract prepared from α-131-20 cells grown in medium A was centrifuged at 200 000 × *g* for 1 h, 76% of the α-naphthyl phosphate phosphatase activity was located in the pellet. After homogenization of the pellet in imidazole buffer and centrifugation at 200 000 × *g* for 1 h, 95% of the activity remained in the pellet. Electrophoresis of the homogenized pellet indicated that it contained only one form of alkaline phosphatase activity, Band I in Fig. 1. Polyacrylamide gel electrophoresis indicated that the activity remaining in the soluble fraction of the extract contains Band II and III phosphatase activity.

To determine the nature of the interaction of Band I with the particulate fraction, the pellet of the 200 000 × *g* centrifugation containing the particulate enzyme was homogenized in buffer containing either 1 M NaCl, 1 M NaBr, 1 M urea, 0.1 M potassium phosphate or 0.1% Triton X-100, and centrifuged at 200 000 × *g* for 1 h. Less than 2% of the activity was released by NaCl, urea, NaBr or potassium phosphate, and approx. 12% was solubilized by 0.1% Triton X-100. Increasing the concentration of Triton X-100 to 1% (w/v) resulted in the solubilization of 20% of the particulate enzyme. These data suggest that the enzyme is bound tightly and does not represent the nonspecific binding of the repressible enzyme (Band III) to some particulate cellular structure.

Chromatography

A crude extract of α-131-20 cells derepressed for 8 h in the absence of phosphate was bound to a DEAE-cellulose column and eluted with NaCl gradient as described by Toh-e et al. [5]. The particulate enzyme, which was identified

by the fact that it was found in the pellet after centrifugation at 200 000 × *g* for 1 h, was not eluted from the column, but remained at the top of the column. The repressible enzyme activity (Band III) and the constitutive *p*-nitrophenyl phosphate specific enzyme (Band II) eluted as previously reported [5]. The absence of elution of the particulate enzyme was most likely due to its particulate nature. Identification of specific enzymes was confirmed by polyacrylamide gel electrophoresis, followed by staining for alkaline phosphatase activities with each of the three substrates, as previously discussed.

Kinetic parameters

The washed particulate enzyme was examined with respect to its kinetic constants. With either 5'AMP or α-naphthyl phosphate as substrate, the enzyme has a broad pH profile, with an optimum at pH 8.5 (Fig. 2). At the pH of 8.5, the enzyme exhibits an apparent K_m of 0.25 mM with respect to *p*-nitrophenyl phosphate and 0.38 mM with respect to α-naphthyl phosphate (Fig. 3). Triton X-100-solubilized particulate enzyme has similar kinetic constants. The soluble repressible phosphatase (Band III on electrophoresis) has an apparent K_m of 0.065 mM with respect to *p*-nitrophenyl phosphate. The catalysis of *p*-nitrophenyl phosphate hydrolysis by particulate phosphatase was inhibited by inorganic phosphate with an apparent K_i of 1.1 mM. 5'AMP, the most rapidly hydrolyzed natural substrate identified for the particulate phosphatase, was also used for kinetic analysis. The particulate phosphatase exhibited an apparent K_m of 1.0 mM for AMP. Similar kinetic constants were obtained for Trinton X-100-solubilized particulate enzyme. The soluble repressible enzyme exhibited a K_m of 0.3 mM for AMP.

A variety of substrates were examined at a concentration of 2 mM as possible substrates for the particulate phosphatase. As can be seen in Table I, the particulate enzyme attacked a variety of monophosphorylated sugars, but had no effect on cyclic nucleotides and no detectable activity on pyrophosphate or polyphosphate. From examination of the hydrolysis of AMP, ADP and ATP, it

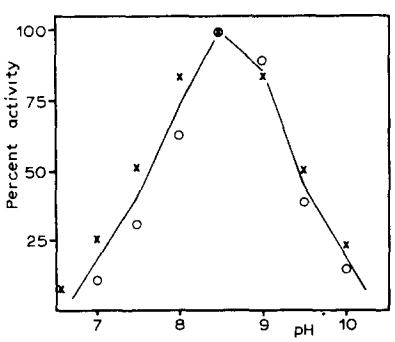

Fig. 2. Effect of pH on particulate alkaline phosphatase activity. Washed particulate enzyme was incubated at various pH values, utilizing either 5'AMP (X) or α-naphthyl phosphate (○) as substrate. The activity is expressed as percent of maximal activity with each substrate.

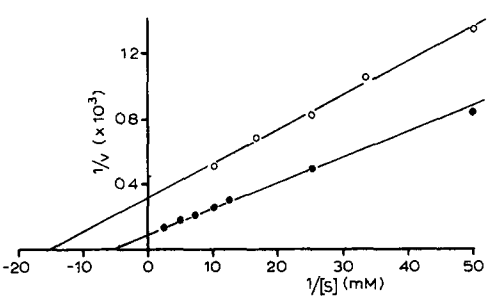

Fig. 3. Effect of substrate concentration on particulate alkaline phosphatase activity. Washed particulate enzyme (●——●) or purified soluble repressible enzyme (○——○) were incubated with various concentrations of *p*-nitrophenyl phosphate and assayed. Data are presented in a Lineweaver-Burk plot.

TABLE I

SUBSTRATE SPECIFICITY OF PARTICULATE ALKALINE PHOSPHATASE

Washed particulate alkaline phosphatase (5 mg protein) was incubated with 2.0 μmol substrate in 1 ml 250 mM Tris-HCl buffer (pH 8.5)/10 mM $MgSO_4$/10 μM $ZnSO_4$. After 10 min, the assay mixture was measured for inorganic phosphate as described in Materials and Methods. Substrate and enzyme blanks were run for each sample.

Substrate	Relative activity
p-Nitrophenyl phosphate	100 nmol/min
Pyrophosphate	<0.5
Tripolyphosphate	<0.5
Tetrapolyphosphate	<0.5
Glucose 6-phosphate	19
Fructose 6-phosphate	10
Mannose 6-phosphate	11
Histidinyl phosphate	43
Adenosine 5′-monophosphate	120
Adenosine 5′-diphosphate	21
Adenosine 5′-triphosphate	4
cyclic GMP	<0.5
cyclic AMP	<0.5

appears that increasing the number of phosphate residues in the sequence decreases the rate of hydrolysis by the enzyme.

Stability and heat inactivation

Both the washed particulate and the repressible soluble alkaline phosphatase were examined with respect to their inactivation by heat. The results of this study are presented in Fig. 4. Similar results were obtained for both Triton X-100-solubilized and crude particulate phosphatase.

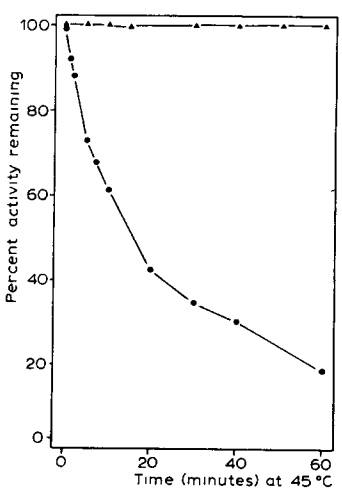

Fig. 4. Heat inactivation of particulate and soluble repressible alkaline phosphatase. Washed particulate alkaline phosphatase ((▲———▲) and partially purified repressible alkaline phosphatase (100 μmol/min) (mg protein) (●———●) were incubated at 45° C. Activity is expressed as the percent of initial activity remaining.

The pH stabilities of washed particulate and soluble derepressible enzymes were examined from pH 4.0 to 8.0 in the presence of 2 mM $MgSO_4$/10 μM $ZnSO_4$/0.5 mM $MnSO_4$ or 1 mM EDTA. The results of this study are presented in Fig. 5.

Physiological studies

The concentration of particulate and soluble α-naphthyl phosphate phosphatase activity was determined in a variety of strains grown under different physiological conditions. The specific activities of both particulate and soluble α-naphthyl phosphatase were examined during derepression of α-131-20 strains in 1% phosphate-free glucose medium (Fig. 6A). The soluble enzyme increased approx. 25-fold in specific activity, whereas the specific activity of the particulate enzyme demonstrated no significant change. Cells derepressed in 1% glucose supplemented with Wickerham's minimal medium minus phosphate, exhibited similar kinetics of depression (unpublished data). The protein content of these cells decreased approx. 30% during 8 h of derepression in 1% glucose medium.

When extracts of different strains, grown in high phosphate medium, were examined for α-naphthyl phosphate phosphatase activity, it was found that the particulate enzyme was present in all of the strains examined. The particulate enzyme had a similar specific activity ($11 \cdot 10^{-3}$—$27 \cdot 10^{-3}$ μmol/min per mg protein in each strain, except for the phosphatase mutants A137 and Al-14-1D

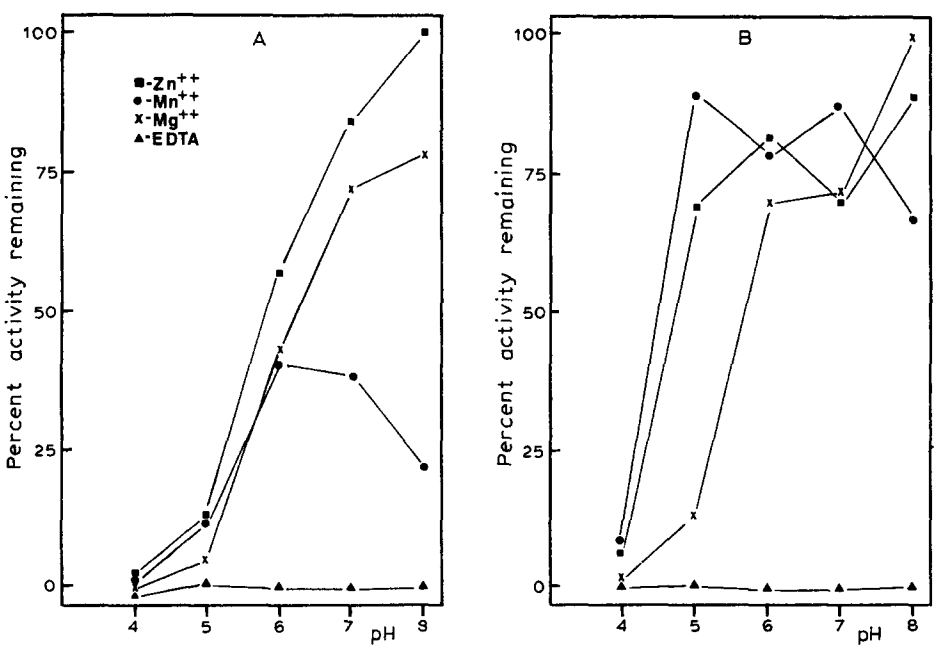

Fig. 5. pH stability of particulate and soluble alkaline phosphatases. Purified repressible alkaline phosphatase (A) and washed particulate enzyme (B) were incubated at various pH values with either 0.01 mM $ZnSO_4$ (■———■), 0.5 mM $MnSO_4$ (●———●), 2.0 $MgSO_4$ (x———x) or 1 mM EDTA (▲———▲). Activity is expressed as the percent of original remaining after 8 h incubation.

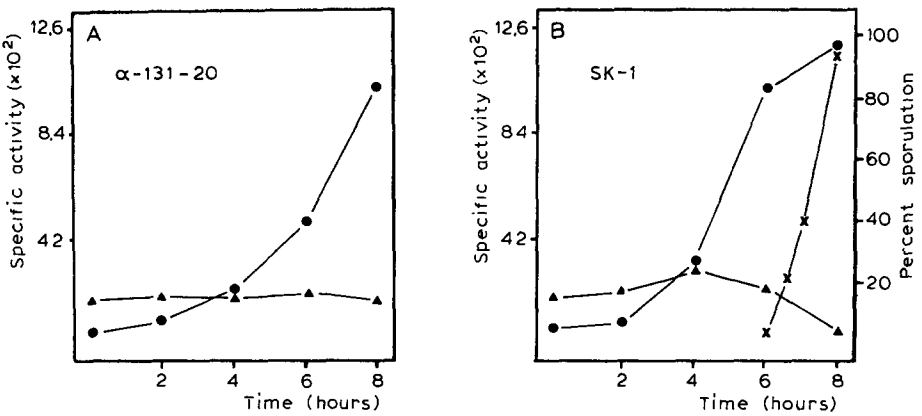

Fig. 6. Levels of alkaline phosphatase during incubation in phosphate free media (A) and during sporulation (B). Cells (α-131-20), grown in YPD medium, were resuspended in 1% glucose (A) and assayed at various times for particulate alkaline phosphatase (▲———▲) or soluble repressible alkaline phosphatase (●———●). Cells (SK-1), incubated in sporulation medium (B), were assayed for particulate alkaline phosphatase (▲———▲), soluble repressible alkaline phosphatase (●———●) and sporulation (x———x). α-Naphthyl phosphatase was utilized as the substrate for alkaline phosphatase.

(Table II). Under the same conditions, the repressible enzyme exhibited a specific activity of $2.8 \cdot 10^{-3}$—$10 \cdot 10^{-3}$ μmol/min per mg protein, except in strains A137 and AL-14-1D. After derepression in 1% glucose for 8 h, the specific activity of the soluble repressible enzyme increased 17—26-fold, whereas the specific activity of the particulate enzyme usually decreases approx. 40% (Table II).

In ts-136 (*rna 1—1*) grown at permissive temperatures (25°C), the repressible alkaline phosphatase could be derepressed approx. one-sixth as much as the wild type strain, whilst the specific activity of the particulate phosphatase did not change (Table II). Strain A1-14-1D (*phoH*) had less than $0.04 \cdot 10^{-3}$ μmol/min per mg protein of the repressible α-naphthyl phosphate phosphatase activity

TABLE II

SPECIFIC ACTIVITY OF α-NAPHTHYL PHOSPHATE PHOSPHATASE IN VARIOUS MUTANT STRAINS OF *SACCHAROMYCES CEREVISIAE*

Cells were grown on YPD medium and either collected immediately or resuspended in 1% glucose for 8 h. Extracts were prepared and phosphatase assayed as described in Materials and Methods. Activity is expressed as μmol α-naphthyl phosphate hydrolyzed/min (mg protein) in the crude extract.

Strain	Specific activity in high P_i medium ($\times 10^3$)		Specific activity in P_i-free medium ($\times 10^3$)	
	Repressible	Particulate	Repressible	Particulate
α-131-20	2.8	13.8	100	15.2
LB60-5A	4.7	27.0	89	8.3
X2180-1B	10.4	24.2	131	8.3
SK-1	6.4	18.6	112	11.7
rna 1-1 (ts 136)	3.0	11.0	9.7	9.0
AL-14-1D (*phoH*)	<0.04	0.48	<0.04	0.48
A137 (*pho 80*)	36.6	49.7	108	15.2

when grown in either repressing or derepressing conditions. This value represents the limit of detection with our assay system. The particulate enzyme activity, although decreased, was approx. one-thirtieth the specific activity observed in wild type cells.

Strain A137 (*pho 80*), which was isolated as a constitutive acid phosphatase mutant, had a very high level of both soluble and particulate enzyme when grown in YPD medium. When the cells were derepressed on 1% glucose, the specific activities of both enzymes were changed to the levels observed in wild type derepressed strains.

The concentration of particulate and soluble α-naphthyl phosphatase were measured during sporulation of strain SK-1. The soluble repressible enzyme increased 8-fold in 8 h, but the particulate enzyme did not change appreciably (Fig. 6B). The soluble enzyme appears to be the form of alkaline phosphatase previously reported to increase during sporulation [20].

Localization of enzyme

In an effort to identify the intracellular location of the particulate alkaline phosphatase, yeast vacuoles were isolated and examined for particulate phosphatase activity. The data in Table III indicates that the particulate enzyme is a vacuolar enzyme, since it increases approx. 50-fold in specific activity, similar to the vacuolar marker enzymes protease B and α-mannosidase. The low specific activity of succinate dehydrogenase and glucose-6-phosphate dehydrogenase in this fraction indicates the purity of the vacuoles. When the tonoplast membrane was isolated by lysis of the vacuoles followed by centrifugation, the activity of the particulate enzyme and α-mannosidase both increased in specific activity approx. 2-fold, suggesting that the particulate enzyme, as α-mannosidase [23], is associated with the tonoplast membrane. The yield of α-mannosidase and particulate alkaline phosphatase in vacuoles is approx. 25%, indicating that the particulate phosphatase, similar to α-mannosidase [28] is primarily localized in the vacuole.

TABLE III

INTRACELLULAR LOCALIZATION OF PARTICULATE ALKALINE PHOSPHATASE

Fractions were prepared and enzymes assayed as described in Materials and Methods. The specific activity of each enzyme is compared to its specific activity in the crude extract of the spheroplast fraction.

Fraction	Relative specific activities				
	Particulate alkaline phosphatase	α-Mannosidase	Protease B	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase
Spheroplasts	1.0	1.0	1.0	1.0	1.0
Vacuoles	50	40	40	0.01	0.14
Vacuolar membranes	115	87	0.4	0.003	0.02

Discussion

The results of this study indicate that the particulate alkaline phosphatase described herein represents a previously unidentified form of alkaline phosphatase in *S. cerevisiae*. In contrast to the other forms of alkaline phosphatase in this microorganism [1—5], this was a particulate enzyme which was sedimented when centrifuged for 1 h at 200 000 × *g*. The enzyme was also distinguished from the other alkaline phosphatases on the basis of its electrophoretic mobility in polyacrylamide gels, substrate specificity, kinetic parameters, heat inactivation, physiological changes during growth and sporulation and mutant analysis. The particulate alkaline phosphatase was most likely not observed in previous studies because these investigators removed all particulate matter from their extracts prior to detailed analysis [3—5].

A particulate alkaline phosphatase in yeast was reported during the early work with phosphatases in *Saccharomyces carlsbergensis* [19]. These workers suggested that the particulate form was identical to the soluble alkaline phosphatase in this yeast and they attributed the increased percentage of soluble activity in stationary phase cultures to a shift in distribution of the particulate enzyme form. However, since the specific activities of the particulate and soluble forms were not examined, the increased percentage of the soluble form may have represented derepression of a repressible alkaline phosphatase. An examination of the specific activity of the particulate and soluble forms of *S. cerevisiae*, indicates that the level of particulate alkaline phosphatase varies little under a variety of physiological states, including conditions such as phosphate starvation and sporulation, during which the repressible enzyme can vary 10—20-fold.

Further differentiation between the particulate and soluble phosphatase activities was provided by examining several yeast mutants (Table II). Ts-136 (*rna 1—1*) appears to be partially deficient in the derepression of the repressible alkaline phosphatase, as has previously been reported [21]. The particulate enzyme activity remains at the same levels after growth in either the presence or absence of phosphate. In *phoH* (AL-14-1D), which was isolated as a repressible alkaline phosphatase negative strain [5], the repressible enzyme activity was at the limit of detection in cells grown in either repressed or derepressed conditions. The particulate enzyme activity is decreased approx. 30-fold in this mutant. Although the *phoH* mutation affects both activities, it clearly affects the soluble activity to a greater degree than the particulate enzyme. The significance of this difference is unknown, since it is not known whether *phoH* represents a structural or regulatory mutant [5].

Strain LB60-5A was examined to evaluate the role of carboxydrate side chains on these enzyme activities and their distribution. This strain is defective in the synthesis of glucosamine which is required for glycoprotein synthesis [22]. The repressible enzyme is a glycoprotein (Fonzi and Opheim, unpublished data). Derepression of alkaline phosphatase in this strain in the absence of exogenously added glucosamine has little effect on the distribution of the α-naphthyl phosphatases or the depression of active repressible enzyme. This suggests that the carbohydrate moiety may not be responsible for the distribution or synthesis of active, soluble repressible enzyme.

Recently, another alkaline phosphatase has been reported in the protoplasts of *S. cerevisiae*. This enzyme is very active against histidinyl phosphate (150% of the rate of *p*-nitrophenyl phosphate), is inhibited by 50 μM $CaCl_2$, and is absent in phosphate limited cells [27]. The particulate enzyme reported herein is not as active on histidinyl phosphate as *p*-nitrophenyl phosphate, is not inhibited by 2 mM $CaCl_2$ (Mitchell and Opheim, unpublished data) and changes little in concentration during phosphate limitation.

The function of the particulate alkaline phosphatase in metabolism is unknown. It is apparent that this enzyme constitutes a considerable fraction of the alkaline phosphatase in phosphate repressed cells, and it has a number of kinetic and physiological properties which differentiate it from the other alkaline phosphatases. Its substrate specificity suggests that it is not involved in pyrophosphate or polyphosphate degradation. Because of its subcellular distribution, it may be possible that it represents either a precursor or processed form of the repressible alkaline phosphatase, which is also in the vacuole (Ref. 24, Fonzi, Atkinson, Opheim, unpublished data). An analysis of this question requires more extensive genetic and chemical analysis of these two enzyme forms. This work is in progress.

Acknowledgements

These studies were supported in part by Grant A683 from the Robert A. Welch Foundation and by Public Health Service Grant 1 R01 GM 25528-01 from the National Institutes of Health. We wish to thank Nigel Atkinson for purifying yeast vacuoles for these studies.

References

1 Suomalainen, H., Linko, M. and Oura, E. (1960) Biochem. Biophys. Acta 37, 482—490
2 Schurr, A. and Yagil, E. (1971) J. Gen. Microbol. 65, 291—303
3 Attias, J., Bonnet, J.L. and Sauvagnargues, J.C. (1970) Biochim. Biophys. Acta 212, 315—321
4 Gorman, J. and Hu, A.S.L. (1969) J. Biol. Chem. 244, 1645—1650
5 Toh-e, A., Nakamura, H. and Oshima, Y. (1976) Biochim. Biophys. Acta 428, 182—192
6 Hopper, A.K., Magee, P.T., Welch, S.K., Friedman, M. and Hall, B.D. (1974) J. Bacteriol. 119, 619—628
7 Kane, S.M. and Roth, R. (1974) J. Bacteriol. 118, 8—14
8 Novick, P. and Schekman, R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1858—1862
9 Roth, R. and Halvorson, H.O. (1969) J. Bacteriol. 98, 831—832
10 Opheim, D.J. (1978) Biochim. Biophys. Acta 524, 121—130
11 Gancedo, J. and Gancedo, C. (1971) Arch. Microbiol. 76, 132—138
12 Racker, E. (1955) Methods Enzymol. 1, 500
13 Juni, E. and Heym, G. (1968) Arch. Biochem. Biophys. 127, 89—100
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Laemmli, U.K. (1970) Nature 227, 680—685
16 Schwencke, J., Magana-Schwencke, N. and Laporte, J. (1977) Ann. Microbiol. 128A, 3—18
17 Matile, P. and Wiemken, A. (1967) Arch. Mikrobiol. 56, 148—155
18 Wiemken, A. (1975) In Methods in Cell Biology, XII, (Prescott, D.M., ed.), pp. 99—109, Academic Press, New York
19 Tonino, G.J.M. and Steyn-Parve, E.P. (1962) Biochim. Biophys. Acta 67, 453—469
20 Opheim, D.J. (1979) J. Bacteriol. 138, 1022—1025
21 Elorza, M.V., Rodriquez, L., Villanueva, J.R. and Sentandreu, R. (1978) Biochim. Biophys. Acta 521, 342—351
22 Whelan, W.L. and Ballou, C.E. (1975) J. Bacteriol. 124, 1545—1557
23 Van der Wilden, W., Matile, P., Schellenberg, M., Meyer, J. and Wiemken, A. (1973) Z. Naturforsch. 28c, 416—421
24 Bauer, H. and Sigarlakie, E. (1975) J. Ultra. Res. 50, 208—215
25 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
26 Ohnishi, T., Gall, R.S. and Mayer, M.L. (1975) Anal. Biochem. 69, 261—267
27 Onishi, H.R., Tkacz, J.S. and Lampen, J.O. (1979) J. Biol. Chem. 254, 11943—11952
28 Wiemken, A., Schellenberg, M. and Urech, K. (1979) Arch. Micobiol. 123, 23—36