Purification and Properties of Alkaline Phosphatase with Protein Phosphatase Activity from *Saccharomyces cerevisiae*

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Z. Naturforsch. **55c,** 588–593 (2000); received May 25, 1999/January 21, 2000

Alkaline Phosphatase, Protein Phosphatase Activity, *Saccharomyces cerevisiae*

An alkaline phosphatase (ALPase) from *Saccharomyces cerevisiae* strain 257 was purified 345-fold with specific activity of 54 533 nmol × min^{-1} × mg $protein^{-1}$. It was shown to be a dimeric protein (apparent mol. wt. approx. 130 kDa) with optimum activity at pH 8.6–8.8 and good stability at 50 °C. The ALPase was a non-specific enzyme hydrolyzing a wide variety of monophosphate esters. The enzyme showed protein phosphatase activity and this activity was not Mg^{2+} – dependent in contrast to *p*-nitrophenyl phosphate (*p*NPP) activity. The K_m value for pNNP hydrolysis was determined to be 2.2×10^{-5} M. Orthophosphate inhibited the enzyme in a competitive mode with the K_i of 2.3×10^{-4} M. Phosphate transfer of the ALPase is almost zero with all alcohols tested except for Tris.

Introduction

Yeast cells produce a group of enzymes which are involved in phosphate uptake, and include acid and alkaline phosphatases in addition to other related enzymes. Most of them are synthesized under phosphate starvation. These enzymes are functionally similar since they catalyze the same biological reaction but they have different pH optima at acid and alkaline pH, respectively, and have different metabolic roles in the cell. Alkaline phosphatases are the product of two structural genes (*PHO8* and *PHO13*) and are localized in the vacuole (Klionsky and Emr, 1989) . Studies on *S. cerevisiae* phosphatases revealed a number of different alkaline phosphatases. The best characterized *PHO8* alkaline phosphatase (Toh-e *et al.,* 1976; Onishi *et al.,* 1979) is a Mg^{2+}-dependent dimeric protein similar to the non-specific alkaline phosphatases in *Escherichia coli* and in mammalian cells (Janeway *et al.,* 1993). The enzyme, product of *PHO13*, is a monomeric P-non-repressible enzyme and is specific with respect to the substrate, attacking *p*-nitrophenyl phosphate (Attias and Bonnet, 1972) or histidinyl phosphate (Gorman and Hu, 1969) but not other substrates at a significant rate. In this work a non-specific alkaline phosphatase with protein phosphatase activity in *S. cerevisiae* has been demonstrated and characterized. To avoid interference of acid phosphatases, a strain defective of constitutive and repressible forms of acid phosphatases was used.

Materials and Methods

Strain and growth conditions

Saccharomyces cerevisiae strain 257 was kindly provided by Prof. P. Venkov from the Institute of Molecular Biology (IMB), Bulgarian Academy of Science (IMB culture collection #257). It was obtained from Prof. A. Hinnen collection (AH:220, *MATa* leu2–3 leu2–112 his3 trp1 *pho3 pho5*). The strain was cultured in 500 ml Erlenmeyer flasks with 100 ml of the YEPD medium (4% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone and 2% (w/v) glucose) on a rotary shaker at 200 rev min^{-1} at 28 °C.

Preparation of phosphoprotein substrates

Unlabeled phosphohistone II-A from calf thymus (Sigma) and phosphocasein from bovine milk (Sigma) were prepared according to Meisler and Langan and Hemmings, respectively, as described by Christova and Galabova (1998).

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Enzyme assays

Alkaline phosphatase activity was assayed in 20 mM Tris [(hydroxymethyl) amino methane-HCl], pH 8.6, with *p*NPP as substrate as described earlier (Galabova *et al.*, 1993). The absorbance of the *p*-nitrophenylate ion (*p*NP) at 410 nm was measured, using a molar absorption coefficient of 16200 $M^{-1}cm^{-1}$. One enzyme unit (U) was defined as the amount of enzyme releasing 1 nmol *p*-nitrophenol per min. The final volume of the reaction mixture was 0.3 ml. When other substrates were tested (2 mM) the assay mixture contained 0.1 ml of the respective substrate instead of *p*NPP. After incubation for 15 min at 37 °C the reaction was terminated by adding 900 µl of molybdate reagent solution according to the method of Bencini *et al.* (1983). The molar absorption coefficient for the phosphomolybdate complex at 350 nm was 7200 $M^{-1}cm^{-1}$. One unit of enzyme activity was defined as 1 nmol of inorganic phosphate (P_i) liberated from the substrate per min at 37 °C. Phosphoprotein phosphatase activity was assayed as described previously (Christova and Galabova, 1998).

Phosphotransferase activity was assayed in 20 mM Tris-HCl buffer pH 8.6 using *p*NPP as the phosphate donor.

Determination of the pH optimum of the enzyme was performed using *p*NPP as substrate in 50 mM buffers, including Tris-HCl buffer (pH 7.2–9.3), and carbonate-bicarbonate buffer (pH 10).

Polyacrylamide gel electrophoresis (PAGE)

Proteins were analyzed by SDS-PAGE (10% w/v, acrylamide) using the method of Laemmli (1970). The ratio of the distance covered by the enzyme bands to the distance covered by bromophenol blue (electrophoretic mobility, R_f) was measured.

Enzyme purification

All chromatography steps were carried out at 4 °C and all concentrations were made in cellophane bags with 40% (w/v) PEG at 4 °C. Protein content of the pooled fractions was followed spectrophotometrically by measuring the absorbance at 280 nm. Accurate protein content was estimated as described by Bradford with bovine serum albumin as a standard.

The alkaline phosphatase protein was purified from the cell free extract of an exponentially-grown culture (20 h) prepared as follows. The yeast cells were separated by centrifugation at 6000×*g* for 10 min at 4 °C and washed twice with deionized water. The pellet was resuspended in a minimal volume of 20 mM Tris-HCl, pH 8.6 (buffer A, containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml antipain, 2 µg/ml leupeptin, 2 µg/ml chymostatin) and disrupted in a homogenizer. Glass beads and unbroken cells were removed by centrifugation at 3000×*g* (10 min, 4 °C) and cell debris were removed by centrifugation at 12000×*g* (20 min, 4 °C). The supernatant fluid was treated with cold (−25 °C ethanol, 1:2.5 (v/v) respectively). The precipitate was centrifuged, resuspended in buffer A and subjected to overnight dialysis against the same buffer. The dialyzed sample was loaded on a DEAE-cellulose column (1.8× 10 cm) equilibrated with buffer A. Linear NaCl gradient (0–0.6 M, 60 ml) was applied. Fractions of 1.2 ml were collected at a flow rate of 25 ml h^{-1}. The concentrated sample (63 to 73 fractions) was applied at a flow rate of 6 ml h^{-1} to a Sephadex G-150 column (2.5 × 45 cm) pre-equilibrated with buffer A, containing 0.1 M NaCl. Fractions of 1.2 ml were collected and assayed for enzyme activity and those with *p*NPP activity were combined. The column was calibrated beforehand with the following molecular mass markers: catalase (240 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa) and peroxidase (40 kDa). At the final stage, the sample was loaded onto a second DEAE-cellulose column (0.8 × 1.6 cm) at a rate of 20 ml h^{-1} and equilibrated with buffer A, containing 0.1 M NaCl. The column was developed with 60 ml of a linear 0.1–0.6 M NaCl gradient in the initial buffer. Fractions with *p*NPP activity were combined, dialyzed and stored at −10 °C.

Results and Discussion

Purification of the enzyme

The process of purification of the ALPase from *S. cerevisiae* strain 257 on the first DEAE-cellulose is shown in Fig. 1. Two peaks with pNPP activity were obtained, the first one represented specific (*pho 13*) alkaline phosphatase and was described in a separate publication. ALPase from the second

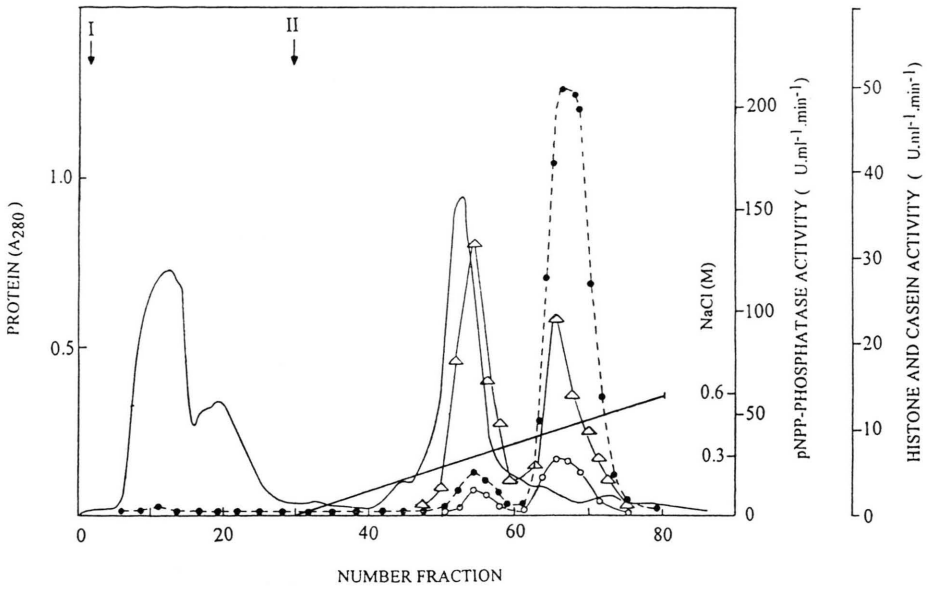

Fig. 1. Elution profiles of phosphatase activities from DEAE-cellulose column. Symbols: — , A_{280}; •—•, *p*NPP phosphatase activity; o—o, casein activity; Δ—Δ, histone II-A activity; I- start of the elution with 20 mM Tris-HCl, pH 8.6; II- start of the salt gradient (0–0.6 M NaCl, 60 ml) in Tris-HCl buffer, pH 8.6.

peak, eluted at 0.35 to 0.45 M NaCl, represented non-specific (*pho 8*) alkaline phosphatase and was used for further purification. At the final stage, a 345-fold purification of the enzyme was achieved with a specific activity of 54 533 nmol×min^{-1}×mg protein^{-1} (Table I).

Molecular mass determination

The molecular mass of the ALPase was estimated by gel filtration and SDS-PAGE. The apparent native molecular mass was determined to be 130 kDa by gel filtration. In SDS-PAGE the enzyme migrated as one band with a molecular mass of 60 kDa (Fig. 2). These results indicate that the ALPase of *S. cerevisiae* strain 257 is a dimeric protein as the other non-specific alkaline phosphatases (Onishi *et al.*, 1979; Janeway *et al.*, 1993).

Catalytic properties of the purified enzyme

Effect of pH and temperature on activity of the ALPase

The effect of pH on enzymatic activity was studied at 37 °C on *p*NPP at various pH values. The activity of ALPase was maximal at pH 8.6–8.8. Non-specific alkaline phosphatases usually have their maximum activity at pH 9.0 (Onishi *et al.*, 1979). Incubation of the enzyme at high temperatures showed that ALPase remained stable at 50 °C. Further increase of the temperature caused

Table I. Purification of ALPase from *S. cerevisiae* #257.

Step	Activity [nmol×min^{-1}]	Protein [mg]	Specific activity [nmol×min^{-1}×mg^{-1}]	Purification (-fold)	Yield (%)
Crude extract	3 843	24.320	158.0	1.0	100.0
Ethanol precipitate	2 882	4.550	633.4	4.0	75.0
DEAE-cellulose	2 738	1.510	1 813.2	11.5	71.2
Sephadex G-100	2 382	0.656	3 631.0	23.0	62.0
DEAE-cellulose	1 308	0.024	54 533.0	345.1	34.1

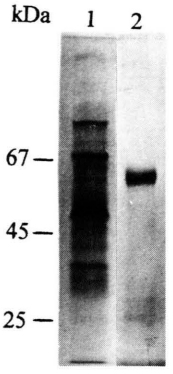

Fig. 2. SDS-PAGE of the ALPase. Samples containing about 60–100 μg protein were loaded onto each lane. Lane 1, molecular mass markers: bovine serum albumin (67 kDa), ovalbumin (45 kDa) and chymotrypsinogen (25 kDa); line 2, crude extract; line 3, purified enzyme.

inactivation and at 70 °C the enzyme retained only 1% of its initial activity after 10 min.

Substrate specificity studies

Several phosphate esters were tested at 2 mM concentration as substrates of the phosphatase activity (Table II). Alkaline phosphatase is capable of hydrolyzing a variety of monophosphate esters. About 60% of *p*NPP activity was obtained with *α*-naphthylphosphate, *o*-phospho-tyrosine and *o*-phosphoserine. Other substrates were hydrolyzed at less than 20% of the rate of *p*NPP hydrolysis.

Table II. Substrate specificity of ALPase.
Phosphate released was determined as described in Materials and Methods. Substrate and enzyme blanks were run for each sample.

Substrate	Activity after second DEAE chromatography	
	P_i production* [nmol min^{-1} ml^{-1}]	Relative activity (%)
*p*NPP	22.00±0.10	100
α-Naphthyl phosphate	14.30±0.45	65
o-Phospho-DL-tyrosine	14.74±0.61	67
o-Phospho-DL-serine	12.54±0.36	57
D-Glucose 1-phosphate	0.66±0.02	3
D-Glucose 6-phosphate	3.28±0.86	15
ATP	2.86±0.06	13
ADP	4.07±0.21	19
3'-AMP	4.81±0.53	22
5'-AMP	4.18±0.46	19
α-Glycerophosphate	0.20±0.07	1
β-Glycerophosphate	3.44±1.66	16
Bis-pNPP	0	0

* Mean values of three different experiments ± SEM are given.

Influence of some effectors on the enzymatic activity

The phosphatase activity was assayed in presence of various effectors (10 mM) listed in Table III. Effectors with pronounced inhibitor influence were tested at 1 mM concentration as well. EDTA was the most powerful inhibitor studied. Activity was greatly affected by the reducing agents L-cysteine and β-mercaptoethanol and their effect was concentration-dependent.

To elucidate the function of the essential metal ions (Zn^{2+}, Mg^{2+}), their ability to restore the phosphatase activity after dialysis against EDTA and deionized water were measured. The EDTA inhibition was not reversed by adding any of the tested Me^{2+} ions. In contrast, Zn^{2+} restored and Mg^{2+} activated the initial activity of alkaline phosphatase dialyzed against water. Probably the difference is due to different "status of damages" of the enzyme in both cases: treating with EDTA is more drastic and causes unreversible changes in the enzyme molecule.

Table III. Effect of some substances on the *p*NPPactivity of ALPase.
The enzyme (2.5 μg in a volume of 1 ml) was pre-incubated at 25 °C for 30 min in the presence of the substances tested, in 50 mM Tris-HCl pH 8.6.

Substance [mM]		pNPP* [nmol min^{-1} ml^{-1}]	Relative activity (%)
None		22.201.12	100
EDTA	10	0.75±0.33	3
	1	<0.20±0.07	<1
o-Phenanthroline	10	0.72±0.23	3
	1	11.56±0.21	52
L-Cysteine	10	1.90±0.89	9
	1	13.30±0.50	59
β-Mercaptoethanol	10	7.04±0.38	32
	1	17.70±0.48	80
Tartrate	10	20.00±0.40	90
Triton X-100	10	24.50±0.50	110
	1	21.50±0.23	97
F^-	10	20.86±0.40	94
Na^+	10	24.86±1.10	112
Mg^{2+}	10	48.40±2.20	218
Mn^{2+}	10	14.60±1.40	66
Zn^{2+}	10	10.65±2.70	48
	1	30.20±0.44	136
Ca^{2+}	10	11.80±1.08	53

* Results are the mean of four replicates ± SEM.

Phosphotransferase activity

The transphosphorylating ability of the phosphatase is studied using *p*NPP as the phosphate donor and some organic compounds containing free hydroxyl groups as phosphate acceptors (Tris, ethanol, glycerol, ethyleneglycol, ethanolamine). In contrast with the high degree of transphosphorylation observed for non-specific alkaline phosphatases (McComb *et al.*, 1979; Onishi *et al.*, 1979), the P_i transfer of the ALPase is almost zero with all tested alcohols except with 1 M Tris. To prevent the influence of Tris concentration, all experiments were carried out using low molarity of the Tris buffer (20 mM).

Determination of kinetic parameters

The K_m of *p*NPP was determined from Lineweaver-Burk plots and Woolf plots. The substrate concentration was varied between 7.5×10^{-6} and 1.5×10^{-4} M. The K_m for *p*NPP hydrolysis was $2.1\pm0.05\times10^{-5}$ M.

The effect of inorganic phosphate concentration on the catalytic properties of the enzyme was determined by comparing the Lineweaver-Burk plots of the enzyme activities at different phosphate concentrations. The K_m value increases with an increase in phosphate concentration which is typical for a competitive mode of inhibition. The K_i of phosphate can be calculated by the equation $K_x = K_m(1 + [P_i]/K_i)$ in which K_x is the apparent K_m in the presence of phosphate. The mean value for K_i of phosphate was calculated to be 2.3×10^{-4} M. Competitive inhibition by phosphate was observed also when Dixon plots were used, with a K_i of $2.1\pm0.2\times10^{-4}$ M.

Table IV. Influence of Mg^{2+} ions on the protein phosphatase activity of the ALPase.
Phosphate released was determined as described in Methods. The assays were performed using 4.6 µg of the purified enzyme in the volume of 1.0 ml.

Substrate	P_i production [nmol $min^{-1}ml^{-1}$]*	
	without Mg^{2+}	with Mg^{2+} (10 mM)
Histone II-A	5.56	5.42
Casein	1.55	1.37
Ser-P_i	49.00	51.80
*p*NPP	50.50	91.00

* Data (three determinations) were pooled to give a mean ±SEM of within 10–15%. Mean values are given.

Protein phosphatase activity

ALPase from *S. cerevisiae* strain 257 exhibited activity on two phosphorylated proteins – casein and histone II-A. As shown by Fig. 1 peaks of enzyme activity on histone II-A and casein are identical with *p*NPPase activity. The activity ratio of casein to histone II-A was 0.28 as shown by Table IV. This value is lower than that for mammalian Mg^{2+}-dependent protein phosphatase (MPP, type 2C) and about two-fold higher than the value reported for MPP-3 from *S. cerevisiae* CBO18 (Murakami *et al.*, 1994). In contrast to dependence of *p*NPP-activity on Mg^{2+} ions, the activity on casein and histone II-A is not Mg^{2+}-dependent as well as the activity on the phosphoamino acids tested. Up to now there are no data about yeast alkaline phosphatases with protein phosphatase activity except for protein phosphatase activity of some bacterial alkaline phosphatases and yeast acid phosphatases (Lau *et al.*, 1982; Lau *et al.*, 1989; Lopandic *et al.*, 1987).

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