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A SPECIFIC ALKALINE *p*-NITROPHENYLPHOSPHATASE ACTIVITY FROM BAKER'S YEAST

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SUMMARY

1. A specific alkaline *p*-nitrophenylphosphatase (*p*-nitrophenylphosphate phosphohydrolase) was purified about 200-fold from commercial baker's yeast (*Saccharomyces cerevisiae*). The enzyme was found to hydrolyse only *p*-nitrophenyl phosphate among several phosphomonoesters at a pH optimum of 8.0–8.2.

2. The enzymatic activity was strongly increased by Mg^{2+}. Ca^{2+}, Zn^{2+} and Be^{2+} were potent inhibitors.

3. EDTA decreased the enzymatic activity by lowering the concentration of Mg^{2+} in the assay medium.

4. Inorganic phosphate was a competitive inhibitor.

5. The enzyme did not show any phosphotransferase activity when Tris was used as an acceptor.

6. A molecular weight of 60 000 was estimated by gel chromatography.

INTRODUCTION

Cellular extracts of baker's yeast (*Saccharomyces cerevisiae*) contain, in addition to a non-specific alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1), an enzyme fraction which catalyses the hydrolysis of *p*-nitrophenyl phosphate at alkaline pH but is without effect on several other phosphomonoesters. This enzyme (*p*-nitrophenylphosphate phosphohydrolase) was recently isolated as roughly purified preparations from cultures of wild type *S. cerevisiae* by Gorman and Hu[1] and from commercial yeast[2]. We describe a partial purification procedure of this enzyme and a study of some of its properties.

MATERIALS AND METHODS

Yeast

Commercial baker's yeast (Lesaffre) was stored at −25 °C until it was used.

Prior to extraction the yeast was washed several times with distilled water until no alkaline phosphatase activity could be detected in the washings.

Enzyme assay

The enzymatic activity was determined by adding 10–50 μl of enzyme solution to 2 ml of 5 mM *p*-nitrophenyl phosphate in 50 mM Tris–HCl buffer (pH 8.2) and 20 mM $MgCl_2$ at 37 °C. The reaction was stopped after 5–15 min by adding 2 ml of 1 M NaOH containing 0.1 M EDTA. Released *p*-nitrophenol was determined at 400 nm against a standard curve of *p*-nitrophenol. Kinetic experiments were performed in a Unicam SP 800 spectrophotometer and recorded at 400 nm. In this case 2.5 ml of substrate were introduced into a 1-cm path length cuvette. One enzyme unit is defined as the amount required to release 1 μmole of *p*-nitrophenol per min.

Methods

Protein concentration was determined by the method of Lowry *et al.*[3] using bovine serum albumin as standard. Inorganic phosphate was determined by the method of Delsal and Manhouri[4]. Phosphotransferase activity was assayed according to the method of Wilson *et al.*[5]. The molecular weight of the enzyme was estimated by gel chromatography according to the method of Andrews[6].

Polyacrylamide gel electrophoreses were performed in 7% gels with a Pleuger "Acrylophor" apparatus in Tris–glycine buffer (pH 8.5) (3 g Tris and 14.4 g glycine per l). No concentration gel was used. A current of 4 mA per tube was applied for 75 min. The gels were stained for alkaline phosphatase activities as described previously[2]. The non-specific alkaline phosphatase bands reacted with both α-naphthyl phosphate and *p*-nitrophenyl phosphate, whereas the specific *p*-nitrophenylphosphatase bands reacted only with the latter substrate.

Glass-distilled deionized water was used throughout. Standard buffer is referred to as 0.1 M Tris–HCl (pH 7.4) and 0.01 M $MgCl_2$.

RESULTS

Purification

1 kg of washed yeast was suspended with 500 ml of 0.2 M $NaHCO_3$ and slowly poured into liquid nitrogen. The frozen yeast was finely ground in a blender with a metal bowl and treated again with liquid nitrogen. The yeast was thawed at room temperature and the suspension was slowly stirred for 8 h. At the end of this period it was centrifuged (30 min, 3000 $\times$ *g*) at 4 °C, and the pellet was washed with 500 ml of 0.2 M $NaHCO_3$.

The pooled supernatants (crude extract) were heated for 5 min at 50 °C, immediately cooled at 0 °C and centrifuged (20 min, 4000 $\times$ *g*) at 4 °C.

The supernatant was subjected to an acetone fractionation. Cold acetone at −25 °C was slowly added under vigorous stirring to a concentration of 45% (v/v). The suspension was maintained for 15 min at −15 °C. It was then centrifuged at −10 °C (10 min, 3000 $\times$ *g*) and the pellet was discarded. The acetone concentration of the supernatant was brought to 60%. The precipitate was collected under the same conditions as above and suspended in standard buffer. Insoluble material was centrifuged off (30 min, 75 000 $\times$ *g*) at 4 °C.

The protein concentration of the solution was adjusted between 10 and 15 mg/ml for a second heat treatment which was conducted in the same conditions as above. It was observed that if the protein concentration was too high, complete inactivation of the enzyme occurred. The next steps were performed at 4 °C.

The supernatant was applied to the top of a DEAE-cellulose column (3.5 cm × 80 cm) which was equilibrated in standard buffer. The enzyme was not adsorbed on the ion exchanger and was eluted by washing the column with standard buffer. The major interest of this step was the removal of a proteolytic activity which was found to inactivate irreversibly the enzyme in further steps of purification if it was not removed.

The active fractions were pooled and subjected to an $(NH_4)_2SO_4$ fractionation. The precipitate from the 0.55–0.65 saturation fraction was collected by centrifugation (20 min, 75 000 × *g*) and resuspended in standard buffer.

The solution was finally applied to the top of a Sephadex G-100 column (2.5 cm × 95 cm) which was equilibrated in standard buffer. The fractions with the highest specific activities were pooled and used for the present study. The overall yield was 23% for a 200-fold purification. The purification procedure is summarized in Table I.

TABLE I

PARTIAL PURIFICATION OF YEAST *p*-NITROPHENYLPHOSPHATASE

Purification step	*Volume (ml)*	*Protein (mg/ml)*	*Activity (μmoles/min per ml)*	*Specific activity (μmoles/min per mg)*
Crude extract	1120	43.7	2.10	0.048
1st heating	1040	42.3	2.20	0.052
45–50% (v/v) acetone	182	31.3	6.85	0.216
2nd heating	520	6.3	2.38	0.374
DEAE-cellulose 0.55–0.65 satn $(NH_4)_2SO_4$	860	2.9	1.23	0.426
Sephadex G-100	52	4.4	16.7	3.76
	60*	0.17	1.76	10.3

* Obtained from 10 ml of the $(NH_4)_2SO_4$ fraction.

The activity of the preparation decreased about 60% after 1 month at 4 °C. The preparation was devoid of specific α-glycerophosphatase activity (2-phosphoglycerol phosphohydrolase, EC 3.1.3.19) (pH optimum 6.5) which had been found in less pure extracts[2]. However, it contained a residual specific inorganic pyrophosphatase activity (pyrophosphate phosphohydrolase, EC 3.6.1.1) (pH optimum 7.5) with about 5% of the original activity in the crude extract.

Gel electrophoreses

The presence of the enzyme in the different purification steps was followed by polyacrylamide gel electrophoresis (Fig. 1). As was previously observed[2], the crude extract showed two bands that reacted with *p*-nitrophenyl phosphate, one of which reacted also with α-naphthyl phosphate. The totality of the non-specific phosphatase

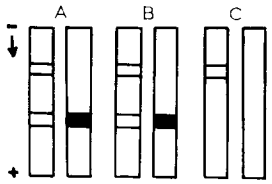


Fig. 1. Polyacrylamide gel electrophoreses of different fractions obtained during purification. After electrophoresis the gels were incubated in solutions of *p*-nitrophenyl phosphate (white bands) and α-naphthyl phosphate (black bands). A, crude extract; B, 0–45% acetone fraction; C, 45–60% acetone fraction.

and a small part of the *p*-nitrophenylphosphatase were precipitated in the 0–45% acetone fraction. The remainder of the latter enzyme was recovered in the 45–60% acetone fraction. All the fractions from the following steps showed one active band on *p*-nitrophenyl phosphate and none on α-naphthyl phosphate. The final preparation showed three major protein bands by staining with amidoschwartz, one of which corresponded to the enzyme.

Specificity and pH optimum

The high specificity of the enzyme for *p*-nitrophenyl phosphate was confirmed with the partially purified preparation on a greater number of substrates. New substrates such as glucose 1-phosphate, threonine phosphate, pyridoxal phosphate and phenyl phosphonate were assayed and were found not to be hydrolyzed by the enzyme (Table II). Moreover, the fraction isolated by Gorman and Hu[1] was inactive on L-histidinol phosphate, 5'-AMP, glucose 6-phosphate and fructose 1,6-diphosphate.

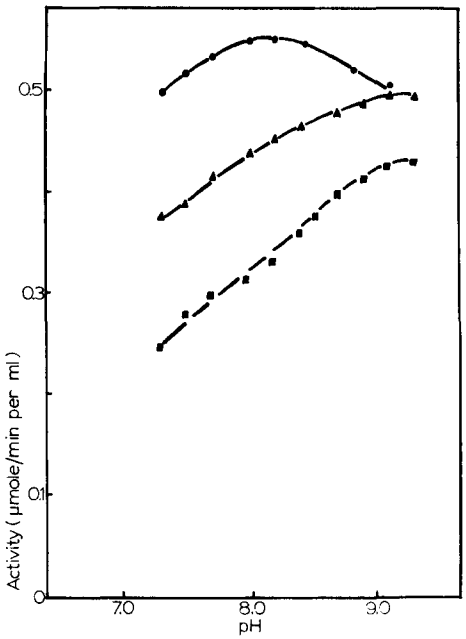
The pH optimum was 8.0–8.2 in 5 mM *p*-nitrophenyl phosphate, 50 mM Tris–HCl buffer and 20 mM Mg^{2+}. When higher concentrations of Tris–HCl were used the pH optimum was shifted to more alkaline values and the activity was decreased (Fig. 2). It is not clear at present whether this phenomenon is due to the increase in ionic strength or to a specific effect of Tris.

TABLE II

SUBSTRATE SPECIFICITY

The substrates were assayed at a concentration of 5 mM in 50 mM Tris–HCl (pH 8.2) and 20 mM Mg^{2+} at 37 °C. The enzymatic reaction was stopped after 20 min. P_i released was determined by comparison with a control which had been incubated without enzyme.

Substrate	*Relative activity*
p-Nitrophenyl phosphate	100
Phenyl phosphate	<1.0
α-Glycerophosphate	<2.0
β-Glycerophosphate	<1.0
o-Phosphoserine	0
Phosphothreonine	<0.5
Phosphoethanolamine	<2.0
Phosphocholine	<1.0
Glucose 1-phosphate	0
Pyridoxal phosphate	<2.0
Phenyl phosphonate	<2.0



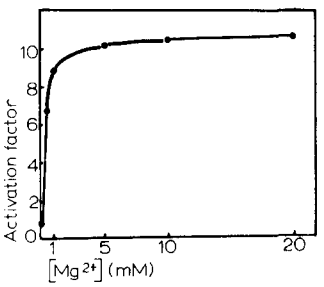


Fig. 2. Effect of pH and Tris concentration on the enzymatic activity. The buffered substrates contained 5 mM *p*-nitrophenyl phosphate and 20 mM $MgCl_2$ at different pH values and molarities of Tris–HCl: 0.05 M (●—●), 0.25 M (▲—▲) and 0.5 M (■—■).

Fig. 3. Activation by Mg^{2+}. The enzyme was assayed in 5 mM *p*-nitrophenyl phosphate and 50 mM Tris–HCl (pH 8.2) in the presence of increasing concentrations of $MgCl_2$. In order to obtain a Mg^{2+}-free enzyme a part of the enzyme solution was filtered through a Sephadex G-25 column which was equilibrated with 0.1 M Tris–HCl (pH 7.4).

Stability

The enzyme was stable when heated for 30 min at 50 °C it was completely inactivated after 20 min at 70 °C. The enzyme was also unstable to acid pH, rapid inactivation occurred below pH 5.

Metal ions

The enzyme was strongly activated by Mg^{2+}, it was almost inactive in the absence of the metal ion. Maximal activation was observed at a concentration of 20 mM Mg^{2+} for which the activity was increased 10–12-fold (Fig. 3).

Other divalent cations showed little activation compared to Mg^{2+}. However, Ca^{2+}, Zn^{2+} and Be^{2+} were strong inhibitors (Table III). Na^+ and K^+ had no effect when the enzyme was assayed in the presence of 20 mM Mg^{2+}.

TABLE III

EFFECT OF METAL IONS

The assay mixture contained 5 mM *p*-nitrophenyl phosphate in 50 mM Tris–HCl (pH 8.2) and the metal ion at a concentration of 1 mM. The addition of the enzyme, which was in a medium containing 10 mM $MgCl_2$ (standard buffer), introduced a concentration of 80 μM Mg^{2+} during the reaction.

Metal ion added	None	Mg^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Ba^{2+}	Sr^{2+}	Mn^{2+}	Ca^{2+}	Zn^{2+}	Be^{2+}
Relative activity	100	288	160	148	130	120	120	68	<1	0	0

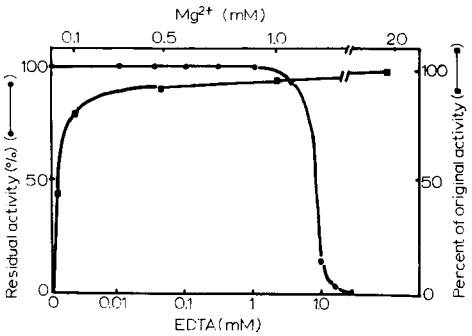
Effect of EDTA

EDTA had no effect at low concentrations when 20 mM Mg^{2+} was present in the reaction mixture. There was a sharp decrease in activity when the concentration of EDTA approached that of Mg^{2+}. The activity was instantaneously restored when excess Mg^{2+} was re-added (Fig. 4).

In the same manner, when the enzyme was incubated for 3 h at 37 °C with a 2-fold excess of EDTA with respect to Mg^{2+} it remained fully active if assayed in the presence of excess Mg^{2+}.

As was observed with crude preparations[2], EDTA had an activating effect at low concentrations when no Mg^{2+} was added to the assay medium. After reaching a maximum, the activity of the enzyme decreased with increasing concentrations of the chelator.

These results suggest that inhibition by EDTA might be due to a lowering in the concentration of free Mg^{2+} in the reaction mixture by chelation rather than to an inactivation of the enzyme. This behaviour towards EDTA seems to be different from that of typical alkaline phosphatases, the inhibition of which is time and concentration dependent[7].



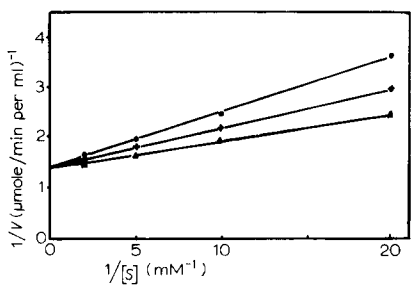


Fig. 4. Inhibition by EDTA and reactivation by Mg^{2+}. The enzyme was assayed in 5 mM *p*-nitrophenyl phosphate, 50 mM Tris–HCl (pH 8.2) and 20 mM $MgCl_2$ in the presence of increasing concentrations of EDTA (●—●). The reverse reaction was studied by adding increasing concentrations of $MgCl_2$ to the medium containing 20 mM EDTA (■—■).

Fig. 5. Competitive inhibition of *p*-nitrophenyl phosphate hydrolysis by inorganic phosphate. Reaction medium: 50 mM Tris–HCl (pH 8.2), 20 mM $MgCl_2$ with different concentrations of *p*-nitrophenyl phosphate. ▲—▲, no P_i added; +—+, 1 mM P_i; ●—●, 2 mM P_i.

Kinetic parameters

The Michaelis constant was determined by the method of Lineweaver and Burk[8]. A mean value of 37 μM was found.

Inorganic phosphate was a competitive inhibitor (Fig. 5). The K_i value determined by plotting $1/V$ *versus* P_i was found to be 2.5 mM. *p*-Nitrophenol, the second reaction product, had no effect on the rate of reaction. Also no inhibition was observed when phenylphosphate was added up to a concentration of 0.01 M, in agreement with the lack of action of the enzyme on this compound.

Transphosphorylation

The enzyme was assayed for phosphotransferase activity. In contrast to clas-

TABLE IV

TRANSPHOSPHORYLATION

Phosphotransferase activity was assayed with the $(NH_4)_2SO_4$ fraction (Table I). The enzyme solution (0.1 ml) was added to 1.0 ml of buffered *p*-nitrophenyl phosphate (5 mM) containing 20 mM Mg^{2+} at 37 °C. The reaction was stopped after 5 min incubation by adding 1.0 ml of 0.5 M H_2SO_4. Orthophosphate (P_i) and *p*-nitrophenol were determined in duplicate on 0.2-ml aliquots.

Medium (pH 8.2)	*p-Nitrophenol (nmoles/ml)*	*P_i (nmoles/ml)*	*p-Nitrophenol / P_i*
0.1 M Tris	311	303	1.03
1.0 M Tris	260	252	1.03

sical alkaline phosphatases[5,9] it was unable to catalyse the transfer of phosphate upon hydrolysis of *p*-nitrophenyl phosphate when Tris was used as an acceptor. As shown in Table IV, equimolecular amounts of *p*-nitrophenol and inorganic phosphate were released. In agreement with this property it can be seen (Fig. 2 and Table IV) that increasing the concentration of Tris did not increase the enzymatic activity as usually observed when transphosphorylation occurs[5], but there was even a decrease.

Molecular weight

The molecular weight of the enzyme was determined by gel chromatography through Sephadex G-200. The column was calibrated with ovalbumin, bovine serum albumin, *Escherichia coli* alkaline phosphatase and human γ-globulin. The elution volume of the enzyme corresponded to a molecular weight of 60 000 (Fig. 6).

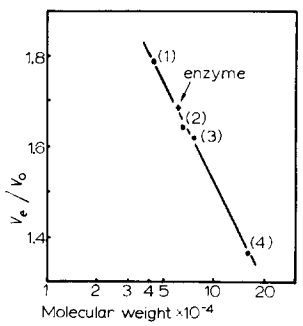


Fig. 6. Molecular weight determination. A column of Sephadex G-200 (2.5 cm × 92 cm) was equilibrated with 0.1 M Tris–HCl (pH 7.4) containing 0.1 M NaCl and calibrated with (1) ovalbumin (45 000), (2) bovine serum albumin (67 000), (3) *E. coli* alkaline phosphatase (80 000) and (4) human γ-globulin (160 000). The elution volumes (V_e) were determined by $A_{280\ nm}$ (1, 2 and 4) or by enzymatic activity (3, enzyme). The void volume of the column (V_0) was determined with a solution of Blue Dextran 2000.

DISCUSSION

We have partially purified, from commercial baker's yeast, an enzyme which catalyses the hydrolysis of *p*-nitrophenyl phosphate at alkaline pH and is inactive on several phosphomonoesters.

The existence of this enzyme was not reported until recently when it was found in wild type cells of *S. cerevisiae* by Gorman and Hu[1] and in commercial baker's

yeast[2]. However, the enzyme is not mentioned in several works on yeast alkaline phosphatase where *p*-nitrophenyl phosphate was used as test substrate[10–12]. Only Stadtman[13] has reported the presence in yeast of an Fe^{2+}-dependent alkaline phosphatase with a pH optimum of about 8.0 which was only active on *p*-nitrophenyl phosphate and phosphoramidate and cocrystallized with alcohol dehydrogenase. This enzyme, like the one described here, released equimolecular amounts of *p*-nitrophenol and inorganic phosphate. It was inactive on creatine phosphate, acetyl phosphate, glucose 1-phosphate, glucose 6-phosphate, β-glycerophosphate, ATP and AMP.

The enzyme may not be present in all strains of *Saccharomyces*. Its biosynthesis may also depend on growth conditions. In extracts where it is present the conditions used may be important for its isolation. It is our experience that the enzyme was very sensitive to organic solvents and to proteolytic enzymes present in the extracts. Acetone fractionation could be achieved by using a short time of contact with the solvent and by operating at very low temperature.

The catalytic effect of the enzyme on the hydrolysis of *p*-nitrophenylphosphate, compared to phenylphosphate for instance, is due to the presence of the nitro group in the para position of the benzene ring. It is well known that the rate of non-enzymatic cleavage of phosphomonoesters is increased by the presence of electron attracting groups on the organic moiety[14]. Indeed, *p*-nitrophenyl phosphate is slowly hydrolyzed at pH 8–10 whereas phenyl phosphate is completely stable[15]. This is not the case, however, for the enzymatic cleavage by alkaline phosphatases where all substrates are split at similar rates[16,17]. Therefore the specificity of the enzyme for *p*-nitrophenyl phosphate cannot be accounted for only on the basis of the electron attractor effect of the nitro group. The fact that orthophosphate is a competitive inhibitor and not phenyl phosphate indicates the importance of both the phosphate and *p*-nitrophenol moieties with the necessary presence of Mg^{2+} for the enzyme to function.

Inhibition by Be^{2+} has been shown by Thomas and Aldridge[18] to be a property common to enzymes which form an intermediate phosphorylated derivative during catalysis. The inhibition of the enzyme by Be^{2+} and the competitive inhibition by orthophosphate are in favor of such a mechanism; they are, however, in apparent contradiction with the lack of phosphotransferase activity of the enzyme. This could be explained by a higher affinity of the phosphoryl-enzyme for water than for Tris during the dephosphorylation step.

Boer and Steyn-Parvé[19] reported that yeast acid phosphatase was also unable to catalyse the transfer of phosphate on Tris upon hydrolysis of *p*-nitrophenyl phosphate. These authors proposed a mechanism of action of the enzyme analogous to the non-enzymatic hydrolysis.

The physiological role of this enzyme is not clear as long as its natural substrate, if any, is not identified. A possibility could be that the enzyme is a molecular variant of the non-specific alkaline phosphatase which would have a much higher affinity for *p*-nitrophenyl phosphate than for the other phosphomonoesters. In relation to this hypothesis it was observed (J. Attias, unpublished) that the molecular weight of the enzyme was about one-half of that of the non-specific alkaline phosphatase. However, we were unable to observe any interconversion of one form into the other.

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