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Purification and partial characterization of acid phosphatase from *Candida lipolytica*

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Non-specific acid phosphatase from *Candida lipolytica* cells was purified 111-fold by chromatography on DEAE-cellulose and gel filtration on Sephadex G-100 and Sepharose 4B. The enzyme is a glycoprotein containing 67% neutral sugars. The molecular mass of the highly purified acid phosphatase was found to be approximately 95 kDa by both SDS-PAGE and gel filtration. The pH and temperature optima were 5·8 and 55 °C, respectively. The enzyme was stable at pH values between 3·5 and 5·5 and at temperatures up to 60 °C. The purified phosphatase had a K_m value of 3·64 mM for *p*-nitrophenyl phosphate and showed broad substrate specificity.

Introduction

In micro-organisms some enzymes are released outside of the cell membrane into the periplasmic space and the cell wall. Acid phosphatase (EC 3.1.3.2), located in the cell wall of yeasts (Arnold, 1981), belongs to this group of enzymes. These enzymes are glycoproteins and their content in yeast cells depends on the phosphate concentration of the growth medium. Although the existence of yeast alkaline phosphatases has been described (Schurr & Yagil, 1971; Onishi *et al.*, 1979), only the acid phosphatases are secreted and these have received the most attention. To investigate the composition and physicochemical and kinetic properties of acid phosphatases, and the genetics of their formation in the yeast cell, it is essential to obtain highly purified preparations of these enzymes.

So far purification and physicochemical characterization of acid phosphatases have been performed for *Saccharomyces cerevisiae* (Schweingruber & Schweingruber, 1982; Barbaric *et al.*, 1984), *Schizosaccharomyces pombe* (Dibenedetto & Teller, 1981), *Candida albicans* (Odds & Hierholzer, 1973), *Rhodotorula rubra* (Watorek *et al.*, 1977), *Rhodotorula glutinis* (Trimble *et al.*, 1981) and *Yarrowia lipolytica* (Lopez & Dominguez, 1988). It has been established that *Candida lipolytica* secretes a number of nucleolytic enzymes such as RNAase, phosphodiesterase, and alkaline and acid phosphatases, (Vasileva-Tonkova & Petkov, 1988).

In this paper, the purification and some properties of an acid phosphatase from *Candida lipolytica* cells are described.

Methods

Yeast strain and growth conditions. Candida lipolytica strain 76-18 was grown in 500 ml Erlenmeyer flasks with 100 ml medium under slow rotatory agitation in a water bath at 24 °C for 24 h. The medium contained 4% (w/v) glucose, 1% (w/v) peptone and 0·2% (w/v) $MgSO_4.7H_2O$. The cell pellet collected was stored at −10 °C until required for enzyme purification.

Enzyme assays. Acid phosphatase activity was assayed with *p*-nitrophenyl phosphate (pNPP) as substrate. The reaction mixture contained 100 µl enzyme sample, 100 µl 0·1 M-sodium acetate buffer (pH 5·5) and 100 µl 3·8 mM-pNPP. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 1 ml 0·2 M-NaOH. The A_{400} was measured. One unit (U) of phosphatase activity was defined as the amount of enzyme catalysing the formation of 1 µmol *p*-nitrophenol min^{-1} under standard assay conditions. When other substrates were used, the method of Lanzeta *et al.* (1979) was applied. One unit of enzyme activity was defined as 1 nmol of inorganic phosphate liberated from the substrate min^{-1} at room temperature.

Phosphodiesterase activity was determined with bis-pNPP as substrate following the same procedure as described for acid phosphatase activity.

Ribonuclease activity was measured as described by Ogata *et al.* (1971) with yeast RNA as substrate.

Enzyme purification. All chromatography steps were performed at 20 °C and all concentrations were made in a cellophane bag containing 40% (w/v) PEG at 4 °C. A crude estimate of the protein content of

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Abbreviation: pNPP, *p*-Nitrophenyl phosphate.

column effluents was made by measuring A_{280}. For accurate measurement of the protein content of the pooled fractions and final enzyme preparation, the Lowry method was used.

Preparation of cell free extract. The yeast cells were separated by centrifugation at 6000 *g* for 10 min at 4 °C and washed twice with cold distilled water. The pellet was resuspended in a minimal volume of extraction buffer (20 mM-Tris/HCl, pH 7·0) and disrupted in a homogenizer. Cell debris and unbroken cells were removed by centrifugation at 12000 *g* for 20 min. The precipitate was washed twice with a small amount of 20 mM-Tris/HCl, pH 7·0. The supernatant fluids were combined and used as a starting material for enzyme purification.

DEAE-cellulose chromatography. The crude extract, after adjusting the pH to 7·0, was directly absorbed onto a DEAE-cellulose column (1·8 × 10 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 7·0. The column was washed with the same buffer until all unbound protein was removed. Elution of the bound acid phosphatase was achieved by changing the pH of the buffer (10 mM-sodium acetate, pH 5·5) followed by a one-step gradient of 1 M-NaCl in the same buffer. Fractions of 1·8 ml were collected at a flow rate of 20 ml h^{-1} and those rich in acid phosphatase activity were collected and concentrated to 1·2 ml.

Sephadex G-100 gel filtration. The concentrated sample was then applied to a Sephadex G-100 column (1·2 × 30 cm) and eluted with 10 mM-sodium acetate, pH 5·5, at a flow rate of 6 ml h^{-1}. Fractions of 1·8 ml were collected and monitored for A_{280} and phosphatase activity. Peak fractions containing the enzyme (9 ml) were combined and concentrated to about 1 ml.

Sepharose 4B gel filtration. The concentrate was applied to a Sepharose 4B column (1·2 × 17 cm) and eluted with 0·1 M-NaCl in 10 mM-sodium acetate, pH 5·0, at a flow rate of 6 ml h^{-1}. Fractions of 1·8 ml were collected and analysed for A_{280} and phosphatase activity. The active fractions were combined, dialysed against water, concentrated and stored at −10 °C.

Determination of molecular mass. The molecular mass of the highly purified acid phosphatase was estimated by gel filtration on a Sephadex G-100 column (1·2 × 30 cm) calibrated with alkaline phosphatase (140000), ovalbumin (45000), trypsin (23000) and cytochrome *c* (12400). The column was equilibrated and eluted with 10 mM-Tris/HCl, pH 7·0, containing 0·1 M-NaCl.

Both denaturing (SDS) and non-denaturing PAGE were performed on a 12% (w/v) polyacrylamide slab gel. Proteins were visualized by staining with Coomassie brilliant blue R-250. For monitoring the phosphatase activity after electrophoresis, the gel was sliced, macerated in water and left overnight at 4 °C to elute the enzyme. Molecular mass markers were cytochrome *c* (12400), ovalbumin (45000) and bovine serum albumin (67000).

Determination of neutral sugars. Neutral carbohydrates were estimated by the phenol–sulphuric acid method (Dubois *et al.*, 1956) using glucose as a standard.

Chemicals. pNPP was from Sigma; Bis-pNPP, α-naphthyl phosphate, glucose 6-phosphate, AMP, UMP, ATP, DEAE-cellulose and standard marker proteins were from Serva; Sephadex G-100, Sepharose 4B and concanavalin A–Sepharose were from Pharmacia; Bacto-peptone was from Difco; and yeast RNA was from BDH. All other chemicals used were of reagent quality.

Table 1. *Purification of acid phosphatase from C. lipolytica*

The purification procedure was performed three times. Values from one representative experiment are shown.

Step	Total A_{280}*	Total activity (U)	Specific activity†	Purification (-fold)	Yield (%)
Crude extract	187·00	610·0	3·26	1·0	100·0
DEAE-cellulose	8·75	325·0	37·14	11·4	53·3
Sephadex G-100	0·72	93·6	130·00	39·8	15·3
Sepharose 4B	0·14	50·7	362·14	111·0	8·3

* A_{280} × volume.
† Total activity/total A_{280}.

Results

Purification

An acid phosphatase from *C. lipolytica* cells grown in the absence of phosphate was purified using a three-step chromatography procedure and the results obtained are presented in Table 1.

Step 1. Four peaks with acid phosphatase activity were obtained upon anion exchange chromatography on DEAE-cellulose (Fig. 1). The first peak was not retained by the cellulose at pH 7·0 and eluted in the flow-through. Peaks II and III were eluted by a change in pH (5·5). Peak IV was eluted by a one-step gradient with 1 M-NaCl in 10 mM-sodium acetate, pH 5·5. Peaks I, II and IV contained a large amount of other proteins and a small amount of acid phosphatase activity (only 2, 1·9 and 4% of the total activity, respectively). Further purification of these fractions was not attempted. This step resulted in a high degree of purification (12-fold) for peak III with a good yield (53%). The protein of peak III was concentrated and further purified.

Step 2. After gel filtration on Sephadex G-100, the enzyme activity was found in the second peak of proteins eluted from the column (Fig. 2). At this stage, 40-fold purification of the enzyme had been achieved with a yield of 15%.

Step 3. Final purification was performed by chromatography on Sepharose 4B. In this step two protein peaks were eluted and the acid phosphatase activity coincided almost exactly with the second protein peak (Fig. 3). The final yield of enzyme activity was 8%, with a specific activity of 362, representing an approximately 111-fold purification over the crude extract. No ribonuclease or phosphodiesterase activities were contained in this fraction.

Molecular mass determination and carbohydrate content

The molecular mass of the purified enzyme was estimated

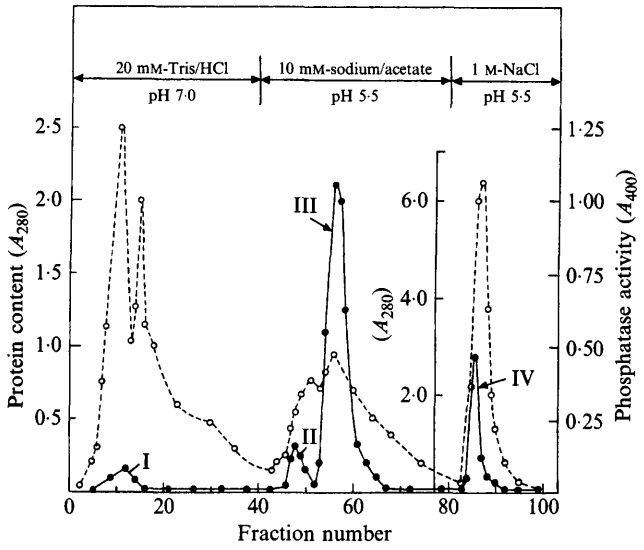

Fig. 1. Ion-exchange chromatography on a DEAE-cellulose column of the acid phosphatases from the cell free extract. Peaks I, II, III and IV represent acid phosphatase activity. Mean data from three chromatograms are shown. ●—●, Phosphatase activity (A_{400}); ○---○, protein content (A_{280}).

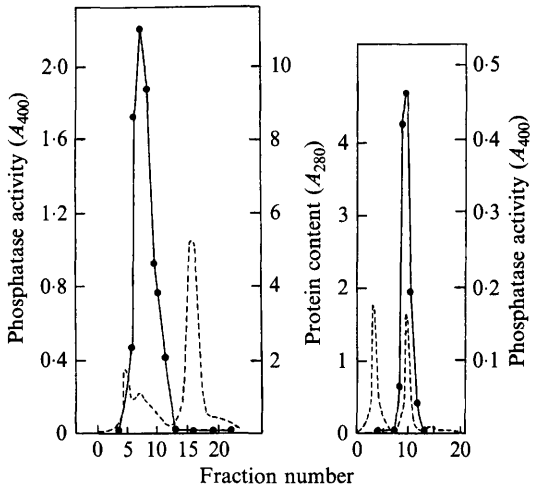

Fig. 2

Fig. 3

Fig. 2. Gel filtration on a Sephadex G-100 column of the acid phosphatase eluted from the DEAE-cellulose column (peak III). ●—●, Phosphatase activity (A_{400}); ---, protein content (A_{280}).

Fig. 3. Chromatography on a Sepharose 4B column of the acid phosphatase eluted from the Sephadex G-100 column. Symbols as for Fig. 2.

to be 95 kDa by gel filtration on Sephadex G-100 and by SDS-PAGE (data not shown).

The mean protein content of the purified enzyme, estimated by the Lowry method, was 33% and the sugar (glucose) content (Dubois *et al.*, 1956) was 67%. The covalent association between carbohydrate and protein was demonstrated by applying the purified enzyme to a small column of concanavalin A–Sepharose. All the activity was retained and could not be eluted with 1 M-α-methylmannoside.

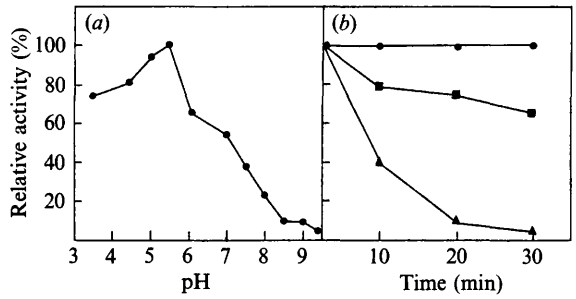

Fig. 4. pH stability (*a*) and thermal inactivation profiles (*b*) of the partially purified acid phosphatase. Preincubation at 50 °C (●), 60 °C (■) and 70 °C (▲). The curves are representative of data from several experiments.

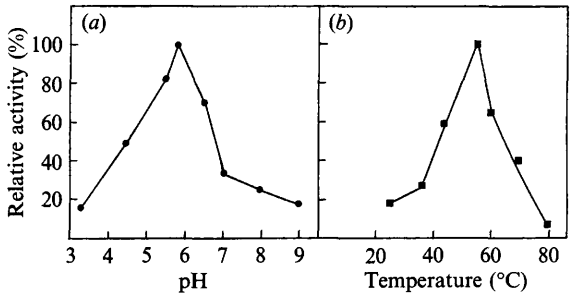

Fig. 5. Effects of pH (*a*) and temperature (*b*) on the activity of highly purified acid phosphatase. The data are representative of three different experiments.

Stability

The final enzyme preparation was incubated at various pH values using 0·1 M-sodium acetate (pH 3·5–6·5) and 0·1 M-Tris/HCl (pH 7·0–9·5) for 18 h at 4 °C. The solutions were then adjusted to pH 5·5 and residual phosphatase activity was measured. The enzyme was stable in a very narrow pH range, from 3·5 to 5·5, and underwent denaturation above pH 5·5; at pH 7·5, 62% loss of activity was observed (Fig. 4*a*).

To check the thermostability of the enzyme, it was incubated in 0·1 M-sodium acetate, pH 5·5, for 10–30 min at various temperatures. Activity was then assayed and expressed as a percentage of the activity of the untreated sample. The acid phosphatase retained its initial activity after 30 min at 50 °C (Fig. 4*b*).

Effects of pH and temperature

Acid phosphatase activity was maximal at pH 5·8 using sodium acetate and Tris/HCl buffers (Fig. 5*a*). The optimal temperature for enzyme activity in the standard assay was 55 °C; 59 and 42% of the maximum activity

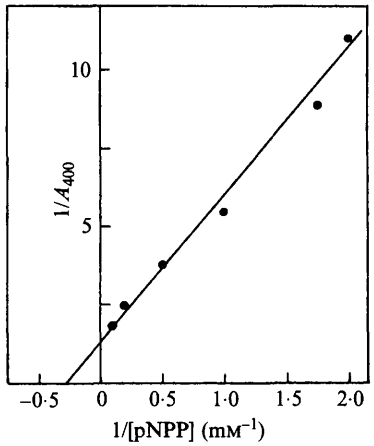

Fig. 6. Effect of pNPP concentration on the activity of acid phosphatase from *C. lipolytica* (Lineweaver–Burk plot).

Table 2. *Substrate specificity relative to pNPP of acid phosphatase from C. lipolytica*

Results are means of three replicates.

Substrate (0·02 M)	Relative rate of hydrolysis (%)
pNPP	100
α-Naphthyl phosphate	100
D-Glucose 6-phosphate	100
ATP	28
AMP	56
UMP	94
Bis-pNPP	0

remained at 45 and 70 °C, respectively, and only 27% was observed at 35 °C (Fig. 5*b*).

K_m and substrate specificity

A Lineweaver–Burk plot for purified phosphatase was constructed with enzyme assays performed at pH 5·5. A K_m value of 3·64 mM with pNPP as substrate was determined (Fig. 6).

Substrate specificity was tested with various compounds under standard conditions (Table 2). The acid phosphatase from *C. lipolytica* is a rather non-specific enzyme as it hydrolyses different phosphate esters regardless of the chemical nature of the leaving group. This is the case for pNPP, α-naphthyl phosphate and glucose 6-phosphate which were the best substrates. The enzyme showed very low activity with ATP and was inactive on the phosphodiester bis-pNPP.

Discussion

In this work we have described a three-step purification procedure with a reproducible yield of 8% leading to a highly purified preparation of an acid phosphatase from *C. lipolytica* cells. Derepression of the acid phosphatase of *C. lipolytica* occurs probably in a similar way to that described for *S. cerevisiae* (Field & Schekman 1980) and for *Y. lipolytica* (Lopez & Dominguez 1988). The enzyme (as in *S. cerevisiae*; Linnemans *et al.*, 1977) is mainly located in the cell wall (data not shown). The enzyme comprised, by total mass, 67% carbohydrate and 33% protein. The high carbohydrate content is characteristic of acid phosphatases in yeasts. The enzyme from *S. cerevisiae* contains 50% carbohydrate (Boer & Steyn-Parve, 1966), from *Sc. pombe*, 66% (Dibenedetto & Cozzani, 1975), from *C. albicans*, 88% (Odds & Hierholzer, 1973), and from *R. rubra*, 57% (Watorek *et al.*, 1977).

It was found by electrophoresis and gel filtration that the native phosphatase from *C. lipolytica* has a molecular mass of 95 kDa. It has been reported that the acid phosphatase from *C. albicans* is a monomer with a molecular mass of 115 kDa (Odds & Hierholzer, 1973), a value similar to that which we obtained for *C. lipolytica*. By native electrophoresis of the purified enzyme, the presence of a single enzymically active band was demonstrated.

The general enzymic properties of the highly purified acid phosphatase from *C. lipolytica*, such as the pH–activity curve, stability to pH, heat denaturation, substrate specificity and K_m value, are similar to those described for acid phosphatases from other yeasts, in particular *S. cerevisiae* (Barbaric *et al.*, 1984), *Sc. pombe* (Dibenedetto & Cozzani, 1975). *C. albicans* (Odds & Hierhozer, 1973) and *Y. lipolytica* (Lopez & Dominguez, 1988). The enzyme from *C. lipolytica* is relatively heat-resistant, similar to the acid phosphatase from *R. rubra* (Watorek *et al.*, 1977).

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