Acid phosphatase of the yeast *Saccharomyces cerevisiae*. Purification and properties of the enzyme

W. WĄTOREK AND B. MORAWIECKA

Department of Molecular Biochemistry, Institute of Biochemistry University of
Wrocław, Wrocław

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Abstract

Acid phosphatase from the yeast *Saccharomyces cerevisiae* was purified to homogeneity as ascertained by ultracentrifugation and electrophoresis. The purification procedure involved mechanical cell disruption, ethanol precipitation, chromatography on DEAE-cellulose, gel filtration on Sepharose 4B. The sedimentation constant $S_{20}^{0.580}$ of the purified enzyme was 15.4 S. Carbohydrate content accounted for 50% of the total molecular weight of the enzyme. The optimum pH for purified enzyme was 3.0–3.5, it was stable at pH 3.0–5.0 at room temperature. After 10 min. incubation at 45°C, 50 per cent of the enzymatic activity was lost. Michaelis constant was found to be 1.3×10^{-4} M for p-nitrophenylphosphate and 5×10^{-4} M for β -glycerophosphate as substrates. The enzyme was inhibited by Hg^{2+} , Cu^{2+} , Fe^{3+} , molybdate, phosphate, arsenate, fluoride ions. Inhibition caused by fluoride ions was noncompetitive, by phosphate — competitive, 5 M urea inactivated the enzyme completely, inactivation was reversible at urea concentration below 2.5 M.

INTRODUCTION

A characteristic trait of many microorganisms is excretion of some enzymes through the cell membrane. To this type of enzymes belongs acid phosphatase of yeasts. It is a glycoprotein localized in the cell wall (Schmidt et al., 1963; Boer and Steyn-Parvé, 1966).

Investigations of this enzyme are mainly concentrated on the mechanism of its biosynthesis, secretion and regulation of the enzymatic activity (Boer et al., 1975; Van Rijn et al., 1972; Günther and Kattner, 1966).

For full elucidation of these problems it is necessary to isolate the enzyme and obtain a better knowledge of the properties of the enzyme of a genetically defined yeast strain. Up-to-date investigations on the purification and properties of acid phosphatase were mainly carried out on

yeasts strains used for industrial processes (Boer and Steyn-Parvé, 1966; Mildner et al., 1976).

Preliminary investigations on acid phosphatase of a genetically well defined yeast strain have been performed by Wątopek (1975).

In the present paper the diploid yeast strain *Saccharomyces cerevisiae* obtained from the cross of the strains D-225-5A and 55-R5-3C was further examined. The aim in view was to isolate and purify acid phosphatase from yeast and to determine its chemical composition, substrate specificity, and the influence of metals and other compound on the enzyme.

MATERIAL AND METHODS

As source of the enzyme served the diploid strain *Saccharomyces cerevisiae* obtained by crossing the strains D-225-5A and 55-R5-3C. They originated from professor Lachowicz's collection (Department of Genetics, Institute of Microbiology, University of Wrocław). The yeast was cultured on minimal medium according to Schurr and Yagil (1971) at 28° C with a limited amount of inorganic phosphate. Cultures in a 4-1. volume were shaken on a WS-2 shaker at 40 rpm in 10-1. flasks with flat bottoms. The culture was interrupted towards the end of the logarithmic phase of growth, when the turbidity of the cells suspension corresponded to extinction $E_{440} = 1.5$.

Growth of the yeast in culture was determined by measuring the turbidity of the cell suspension at wavelength 440 nm in a Spekol colorimeter.

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Total sugars were determined by the phenol method (Dubois et al., 1956) with mannose as standard.

Enzymatic activity was determined at pH 3.0 in 0.1 M glycine-HCl buffer at 30° C in the presence of 3.23 mM sodium p-nitrophenyl-phosphate as substrate. The volume of the reaction mixture was 2.5 ml. The reaction was interrupted by adding 5 ml of 2.0 M NaOH. The amount of released p-nitrophenol was determined by measuring extinction in a Spekol colorimeter or a VSU-2P spectrophotometer at wavelength 410 nm. When other substrates were used, the phosphate released during reaction was determined by the method of Fiske-Subbarow (1925). As unit of enzymatic activity was adopted the release from the substrate of 1 μ M phosphorus during 1 min at 30° C in glycine-HCl buffer pH 3.0. Specific activity was expressed in u./mg protein.

The enzymatic activity of the yeast native cell suspension was determined as follows: the cell sediment obtained after centrifugation of a known volume of the culture was resuspended in 1 ml of glycine-HCl buffer pH. 3.0. A specified amount of the cell suspension in buffer was taken for determinations. The reaction was run at 30° C in the presence of 3.23 mM sodium p-nitrophenylphosphate as substrate. The reaction

mixture volume was 2.5 ml. The reaction was interrupted by addition of 5 ml of 0.2 M NaOH. The yeast cells were removed by centrifugation and the released p-nitrophenol was determined in the supernatant.

The influence of pH on acid phosphatase activity was examined within the range 1.9—8.5. The following buffer systems were applied: citrate-HCl, pH range 1.9—3.0, glycine-HCl, pH range 2.6—3.4, acetate, pH range 3.5—5.5 and tris-maleate-NaOH, pH range 5.4—8.45. The ionic strength of the buffer was adjusted to 0.1 with NaCl.

The influence of pH on the stability of the enzyme was investigated in the above listed buffer systems: 5.9 μ g protein was incubated in 1 ml of the given buffer at room temperature. After 20, 60, 120 and 180 min and 17 h 0.59 μ g of protein was taken and enzymatic activity was determined at pH 3.0.

The influence of temperature on the stability of the enzyme was tested by incubating 0.59 μ g protein in 2 ml of 0.1 M glycine-HCl buffer at 30° C, 40°, 45° and 50° C. Incubation was interrupted after 10, 20, 30, 40, 50 and 60 min by cooling the sample in an ice bath, and enzymatic activity was determined.

Ion exchanger chromatography was run on DEAE-cellulose on a column 3.5 cm in diameter and 35 cm long, equilibrated with 0.01 M acetate buffer pH 4.65. Protein was eluted with an increasing 0.5 M buffer concentration gradient. Fractions of 10 ml were collected at flow rate 1 ml/min.

Molecular filtration on sepharose 4B was done in 0.1 M acetate buffer at pH 4.2 on a column 1.5 cm in diameter and 90 cm long. Fractions of 5 ml were collected at flow rate 0.1 ml/min.

Analytical ultracentrifugation was done on a MOM type 3170 ultra centrifuge with Philpot-Swenson optics at 50 000 rpm ($142 \times 345 \times$ g) at 20° C in acetate buffer pH 3.8 and ionic strength $I = 0.1$.

Electrophoresis was run in polyacrylamide gel pH 9.5 after Smith et al. (1968). On the gel 30 μ g protein was placed. The protein in the gel was stained with a 1 per cent Amide Black solution in 7 per cent acetic acid. Phosphatase activity was detected in the gel after electrophoresis by the method of diazo-coupling in the presence of sodium α -naphthylphosphate and the coupling agent — Fast Blue B.

RESULTS

The conditions were optimal for increase of acid phosphatase activity and culture growth when medium containing 30 g KH_2PO_4 and 40 g glucose/l. was used.

The cells collected towards the end of the logarithmic growth phase and after washing away the medium were suspended in 0.1 M acetate buffer pH 3.8 and disrupted in Braun's mill with glass beads 0.5 mm in diameter. The homogenate was centrifuged at $20\,000 \times$ g at 4° C for 45

min. The supernatant was dialysed into 0.1 M acetate buffer pH 3.8. The dialysate was precipitated with cold ethanol to a 50 per cent ethanol concentration in the end volume. Phosphatase activity was present in the precipitate. The precipitate was dialysed after dissolution into 0.01 M acetate buffer pH 4.65 and placed on a DEAE-cellulose column equilibrated with the same buffer. Protein was eluted from the column with a linear buffer concentration gradient up to 0.5 M. Fig. 1 shows protein separation and enzymatic activity from DEAE-cellulose. Phosphatase activity was located in the distinctly separated second protein peak. Fractions of this peak were combined and precipitated with ethanol. The sediment after

Fig. 1. DEAE-cellulose chromatography of proteins from *Saccharomyces cerevisiae*. Experimental conditions are described in the text. — protein E_{280} ; --- phosphatase activity u./ml

Fig. 2. Sepharose 4B gel filtration of acid phosphatase fraction obtained from DEAE-cellulose column. Experimental conditions are described in the text. — protein E_{280} ; --- phosphatase activity u./ml

dissolution in 0.1 M acetate buffer pH 4.2 was subjected to molecular filtration on a column filled with sepharose 4B. As seen in Fig. 2, during filtration on sepharose 4B the material separated into two protein fractions. In both phosphatase activity was found. The protein of the first peak, when subjected to analytical ultracentrifugation in 0.1 M acetate buffer pH 3.8 with ionic strength 0.1 was found to be nonhomogeneous (Fig. 3), while the protein of the second peak subjected after sepharose 4B treatment to ultracentrifugation was homogeneous under the same conditions. Its sedimentation constant was $s^0 = 15.4 \times 10^{-13}$ sec (Fig. 3). The homogeneity of acid phosphatase obtained in the second peak after sepharose 4B was also confirmed by electrophoresis in polyacryl gel at pH = 9.5. Fig. 4 shows the presence of a single band staining with Amide Black and exhibiting phosphatase activity.

Fig. 3. Ultracentrifugal patterns of protein fractions obtained after gel filtration on Sepharose 4B

- A — I peak after Sepharose 4B. The protein concentration was 5,9 mg per ml 0,1 M acetate buffer pH 3,8 with NaCl (ionic strength = 0,1)
 B — II peak after Sepharose 4B. The protein concentration was 5,8 mg per ml 0,1 M acetate buffer pH 3,8 with NaCl (ionic strength = 0,1)

Photographs taken 60 min after the rotor had reached 50000 rpm at 20°

Fig. 4. Separation of acid phosphatase from *Saccharomyces cerevisiae* on 7,5% acrylamide gel at pH 9,5. Experimental conditions are described in the text

The weight ratio of sugar to protein in the first peak after sepharose 4B was 1.75 : 1.0, in the second peak this ratio was equal to one.

The results of the successive steps of isolation and purification of acid phosphatase from *Saccharomyces cerevisiae* are shown in Table 1. According to the procedure described, a 12-fold purification of the enzyme was achieved as compared with the crude extract. The yield of the preparation was about 20 per cent.

In the further step of the studies the catalytic properties of acid phosphatase from *Saccharomyces cerevisiae* were closer characterized. The material for examination was the homogeneous protein from the second peak obtained by molecular filtration on sepharose 4B.

Table 1
Isolation and purification of acid phosphatase from *Saccharomyces cerevisiae*

Steps of purification	Protein mg	Total activity μ	Specific activity μ /mg protein	Purification factor	Recovery %
Homogenate	5880	32340	5,5	1,0	100,0
Ethanol precipitate	663	15300	23,0	4,0	47,3
DEAE-cellulose	227	9990	44,0	8,0	31,0
Sepharose 4B II peak	79	5370	67,8	12,3	16,6

It was found that the optimum pH for acid phosphatase activity lies between 3.0 and 3.5. The relation between the enzymatic reaction rate and pH is shown in Fig. 5.

The effect of pH on preservation of the activity of acid phosphatase from *S. cerevisiae* (Fig. 6) was investigated. Enzymatic activity was unchanged within a narrow range of pH from 3.0 to 5.0. Below and above this pH range the enzymatic activity gradually decreased within 17 h of the experiment. At pH 2.0 and 7.8 the enzyme was immediately inactivated.

Fig. 7 presents the effect of temperature on preservation of activity of acid phosphatase from yeast. Purified phosphatase is a thermolabile enzyme. During 10 min of incubation at 45° C enzyme activity diminished by one half, at 50° C it completely disappeared.

In order to establish the substrate specificity of purified acid phosphatase, the activity of the enzyme was tested in the presence of aromatic and aliphatic phosphate esters, some nucleotides, ATP, pyrophosphate and bis-p-nitrophenylphosphate (Table 2). The enzyme hydrolyses both artificial substrates such as p-nitrophenylphosphate, phenylphosphate, α - and β -naphthylphosphate and nucleotides and phosphate esters of su-

gars. The highest catalytic activity was demonstrated in the presence of phenylphosphate.

Fig. 5. Effect of pH on *Saccharomyces cerevisiae* acid phosphatase activity. Experimental conditions described in text

Fig. 6. Rate of inactivation of *Saccharomyces cerevisiae* acid phosphatase in various buffer solutions at the indicated pH values. Experimental conditions described in text

Figs 8 and 9 show the effect of p-nitrophenylphosphate and β -glycerophosphate on the enzymatic activity of acid phosphatase. K_m of the examined enzymes is 1.3×10^{-4} M in the presence of p-nitrophenylphosphate and 5×10^{-4} M in the presence of β -glycerophosphate as substrate.

Fig. 7. Thermal inactivation of *Saccharomyces cerevisiae* acid phosphatase. Experimental conditions described in text

Fig. 8. Effect of p-nitrophenylphosphate on *Saccharomyces cerevisiae* acid phosphatase activity (Lineweaver-Burk plot)

Fig. 9. Effect of β -glycerophosphate on *Saccharomyces cerevisiae* acid phosphatase activity (Lineweaver-Burk plot)

Table 2

Substrate specificity of *Saccharomyces cerevisiae* acid phosphatase

Substrate 10^{-2} M	Relative activity %
Phenylphosphate	100
P-nitrophenylphosphate	72
α -naphthylphosphate	62
β -naphthylphosphate	52
5' — AMP	42
2'3' — cAMP	4
5' — UMP	56
5' — GMP	38
5' — CMP	51
ATP	55
Glucose-1-phosphate	70
Glucose-6-phosphate	88
Fructose-1, 6-diphosphate	91
Phosphoenolpyruvate	31
6-phosphogluconate	91
α -glycerophosphate	66
β -glycerophosphate	80
Bis-p-nitrophenylphosphate	0
Pyrophosphate	76

The influence of cations on acid phosphatase activity was tested with p-nitrophenylphosphate and β -glycerophosphate as substrate. The results are shown in Table 3. Hg^{2+} and Cu^{2+} ions proved to be inhibitors, the effect of Zn^{2+} , Al^{3+} and Fe^{3+} was weaker. The remaining cations had no noticeable effect on the investigated enzymatic activity.

Table 3

Effect of various cations on *Saccharomyces cerevisiae* acid phosphatase activity

Cation	Relative activity (%)			
	substrate: p-NPP		substrate: β -glycero-P	
	Cation concentration			
	10^{-2} M	10^{-3} M	10^{-2} M	10^{-3} M
Hg ²⁺	8	37	0	46
Cu ²⁺	17	69	25	86
Zn ²⁺	60	97	82	94
Cd ²⁺	100	100	100	100
Ca ²⁺	100	100	100	100
Mg ²⁺	100	100	100	100
Co ²⁺	100	100	100	100
Mn ²⁺	100	100	100	100
Al ³⁺	40	87	35	91
Fe ³⁺			0	89

It was found in further experiments that molybdate, arsenate phosphate and fluoride ions are inhibitors of *S. cerevisiae* acid phosphatase. Compounds breaking disulphide bonds (β -mercaptoethanol and dithiotreitol) as well as chelating substances had no effect on the enzyme activity (Table 4).

Table 4
Effect of various compounds on *Saccharomyces cerevisiae* acid phosphatase activity

Compound added 10^{-3} M	Relative activity %
Na_2MoO_4	0
Na_2AsO_4	0
KH_2PO_4	35
NaF	0
β -mercaptoethanol	100
dithiothreitol	100
p-CMB/ 10^{-4} M/	63
EDTA	100
1, 10-phenantroline	100
α , α' -dipyridyl	100

The results of investigations aimed at determination of the type of enzyme inhibition by phosphate and fluoride ions are illustrated in Figs 10 and 11. The fluoride ion proved to be an incompetent inhibitor of phosphatase activity, whereas phosphate competently inhibited the enzyme activity. K_i for phosphate is 1×10^{-4} M.

Table 5 shows the effect of urea on the activity of *S. cerevisiae* acid

Fig. 10. Effect of p-nitrophenylphosphate on *Saccharomyces cerevisiae* acid phosphatase activity in the presence of fluoride ions (Lineweaver-Burk plot)

Fig. 11. Effect of phosphate ions on *Saccharomyces cerevisiae* acid phosphatase activity, substrate — p-nitrophenylphosphate (Dixon plot)

phosphatase. In a 5 M or higher concentration urea completely inactivates the enzyme. Removal of urea from the reaction medium after preliminary incubation fully reactivates the enzyme if the initial urea concen-

Table 5

Effect of urea on *Saccharomyces cerevisiae* acid phosphatase activity

Urea concentration M	Relative activity (%)	
	in the presence of urea	after the release of urea
10	0	
8	0	0
5	0	28
2,5	34	100
1	79	100
0,5	86	
0,25	89	

tration does not exceed 2.5 M. In the presence of 5 M urea reactivation is partial. The enzyme incubated with 8 M urea could not be reactivated even when the denaturing agent was removed.

DISCUSSION

Biosynthesis and secretion of acid phosphatase from yeast depends on the phosphate and glucose concentration in the medium (Schmidt et al. 1956; Van Rijn et al., 1972). In the present experiments optimal conditions for culture growth and increase of enzymatic activity were 30 mg KH_2PO_4 and 40 g glucose/l. medium concentrations. Toh-e et al.,

(1973) assumed in their studies on yeast acid phosphatase identical phosphate and glucose concentrations as given above as optimal.

The developed method of isolation and purification is simple and allows to obtain in several steps an enzyme preparation with higher specific activity (68 μ ./mg protein) as compared with phosphatase from baker's yeast isolated by Mildner et al. (1976). In the last step of acid phosphatase purification by way of molecular filtration on sepharose 4B the enzymatic activity separated into two peaks. Acid phosphatase occurring in the first peak was heterogeneous in analytical ultracentrifugation and contained 64 per cent of sugar. In the second peak acid phosphatase was homogeneous in ultracentrifuge studies and electrophoresis on polyacrylamide gel, it contained 50 per cent of sugar. Electrophoretic analysis in polyacrylamide gel revealed the presence of a single broad protein band with acid phosphatase activity. The diffuse character of this band suggests microheterogeneity of the enzyme. Odds and Hierholzer (1973) also report diffuse electropherograms of the yeast *Candida albicans* phosphatase. The postulated microheterogeneity may result from protein glycosylation or phosphorylation of various degrees. Dibenedetto and Cozzani (1976) discovered inorganic phosphate covalently bound with acid phosphatase of the yeast *Schizosaccharomyces pombe*.

The sedimentation constant (15.4 S) of the studied acid phosphatase has a value close to that described by other authors for this enzyme (Boer and Steyn-Parvé, 1966; Mildner et al., 1976).

The slight differences in optimum pH, thermostability, pH-stability and K_m values as compared with the data of other authors (Beer and Stein-Parvé, 1966, Mildner et al., 1976) are probably due to the use in the investigations of different yeast strains and culture conditions (mainly different phosphate concentrations in the medium). Touimi-Benjelloun and Bonaly (1975) report different K_m values for acid phosphatase from the yeast *Rhodotorula rubra* cultured on media with various phosphorus content.

The slight effect of agents binding free SH groups on the enzymatic activity lead to the supposition that these groups do not play an essential role in the active centre of the enzyme or that the sugar component makes access to this centre difficult. Similar conclusions are advanced by Mildner et al. (1976).

The influence of urea on the activity of the purified enzyme and the high sedimentation constant suggest the possibility of a subunit structure of the enzyme. This hypothesis finds confirmation in the paper dealing with purification and properties of acid phosphatase from *Schizosaccharomyces pombe* (Dibenedetto and Cozzani, 1975).

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Fosfataza kwaśna drożdży Saccharomyces cerevisiae.
Oczyszczanie i własności enzymu

Streszczenie

Fosfatazę kwaśną izolowano z diploidalnego szczepu *Saccharomyces cerevisiae* otrzymanego przez krzyżowanie szczepów D-225-5A i 55-R 5-3C.

Opracowano metodę izolacji i oczyszczania enzymu opartą na ekstrakcji białka z mechanicznie rozbitych komórek do 0,1 M buforu octanowego pH 3,8, wytrącaniu białka etanolem, chromatografii jonowymiennej na DEAE-celulozie i sędzeniu molekularnym na sefarozie 4B. Homogenność otrzymanego preparatu stwierdzono w elektroforezie w żelu poliakryloamidowym i w ultrawirowaniu. Stała sedymentacji badanego enzymu wynosi 15,4 S. Preparat zawiera 50% cukru. Optimum pH fosfa-

tazy kwaśnej wynosi 3—3,5. Enzym denaturuje się połowicznie po 10 min inkubacji w 45°, trwały jest w wąskim zakresie pH między 3.0 a 5.0. Fosfataza kwaśna z *Saccharomyces cerevisiae* nie wykazuje wybiórczej specyficzności wobec różnych estrów fosforanowych. K_m wobec p-nitrofenylofosforanu wynosi $1,3 \times 10^{-4}$ M a wobec β -glicerofosforanu 5×10^{-4} M. Inhibitorami enzymu są Hg^{2+} , Cu^{2+} , Fe^{3+} , molibdenian, arsenian, fluorek i fosforan. Inhibicja wywołana jonem F^- ma charakter niekompetycyjny, jonem fosforanowym — kompetycyjny. Mocznik w stężeniu 5M i wyższym całkowicie inaktywuje enzym. Inaktywacja ma charakter odwracalny przy stężeniu mocznika mniejszym od 2,5 M.