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PURIFICATION OF PROTOPLAST-SECRETED ACID PHOSPHATASE FROM BAKER'S YEAST

EFFECT ON ADENOSINE TRIPHOSPHATASE ACTIVITY

PAVAO MILDNER, SLOBODAN BARBARIĆ, ŽELJKO GOLUBIĆ * and BLANKA RIES

Laboratory of Biochemistry, Technological Faculty, University of Zagreb, Pierottijeva 6, 41000 Zagreb (Yugoslavia)

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Summary

In order to examine acid phosphatase (EC 3.1.3.2) and ATPase (EC 3.6.1.3) activities of baker's yeast (pH optimum 3.5) a protoplast-secreted enzyme preparation was purified and some physical and chemical properties were studied.

Three protein fractions containing ATPase and acid phosphatase activities, in the same ratio as the initial preparation, were separated by ion-exchange chromatography. The first fraction which had the highest protein content yielded a homogeneous preparation after Sepharose 4B chromatography and was used in further studies.

An attempt to estimate molecular weight of this protein was made.

Attempts to separate acid phosphatase and ATPase activities by ion-exchange chromatography, gel filtration, isoelectric focusing and sucrose density gradient centrifugation have been unsuccessful. Both activities behaved the same way to heat and urea denaturation. These results suggest that the two activities are associated with the same protein molecule.

Introduction

Previous studies from this laboratory [1] suggest that the ATPase (EC 3.6.1.3) activity, encountered in the yeast cell wall is not due to a specific enzyme, but is associated with non-specific acid phosphatase (EC 3.1.3.2).

* Present address: Research Department "Pliva", Pharmaceutical and Chemical Works, Zagreb, Yugoslavia.

Abbreviation: NPh-*P*, *p*-nitrophenylphosphate.

This paper describes the purification of the protoplast-secreted enzyme with the aim of confirming that acid phosphatase and ATPase activities are exerted by the same protein.

Materials and Methods

Preparation of crude enzyme. The yeast *Saccharomyces cerevisiae* (strain 367 Technological Faculty Collection, Zagreb) was cultured aerobically, in phosphate-poor medium [1], by shaking for 12 h in 200 ml batches at 30° C.

The enzyme preparation was obtained from protoplast secretion, and the protoplasts were prepared as described previously [1].

Enzyme assay. Acid phosphatase and ATPase activities were determined under experimental conditions reported earlier [1].

Specific activities of the enzyme preparations were calculated with respect to protein content, determined by the method of Lowry et al. [2] with bovine serum albumin as standard.

Ultrafiltration. In ultrafiltration, a XM 100-A membrane and a Model 202 ultrafiltration cell (Amicon Corp.) was used. The ultrafiltration was carried out under N_2 (2 atm.).

Ion-exchange chromatography. DEAE-Sephadex A-25 was equilibrated with 0.1 M sodium acetate buffer (pH 5.5) and the slurry was poured into a column (0.9 cm × 30 cm).

Elutions were in two stages at a flow rate of 1.5 ml/h. Equilibrating buffer was used as the first eluant and after 19 fractions a linear salt gradient was started (0.0—0.3 M NaCl). Fractions of 1 ml were collected and the change in absorbance at 280 nm was followed using an LKB Uvicord II flow cell.

Sephadex G-200 filtration. The gel, Sephadex G-200, was left to swell in 0.1 M sodium acetate buffer, pH 4.6, then placed into a 1.5 × 30 cm column. The column was operated at a flow rate of 5 ml/h. 1-ml fractions were collected, and absorbance changes at 280 nm were followed.

The same column and the same conditions were used for molecular weight determination of purified enzyme. Proteins for calibration were lactate dehydrogenase, fumarase, catalase, urease and β-galactosidase. During the entire series of experiments the bed height was kept constant. The void volume was determined with a Dextran Blue 2000.

Sepharose 4B chromatography. A column (0.9 cm × 30 cm) was filled with gel, equilibrated with 0.1 M sodium acetate buffer, pH 4.6, and operated at a constant flow rate of 1.5 ml/h. 0.6-ml fractions were collected and tested for protein content and enzyme activities.

Isoelectric focusing. The linear pH gradient for these experiments was established with 1.02% carrier ampholytes, pH 3—10, in an LKB column of a total volume of 110 ml. The focusing process was extended over a 64-h period at a temperature of 15° C, and a potential difference of 300 V. Fractions of 2 ml were collected and subjected to determination of pH, protein content and enzyme activities.

Sucrose density gradient centrifugation. 0.1 ml of purified enzyme solution (300 μg) was layered onto 12 ml of linear sucrose gradient (5—25%) in 0.1 M sodium acetate buffer, pH 4.6. Bovine liver catalase (11.3 S) and bovin serum

albumin (4.6 S) were used as markers. Centrifugation was carried out in 10-h runs with a Beckman ultracentrifuge Model L3-50 at 38 000 rev./min (3° C). Upon completion of the run, 36 fractions of 15 drops each were collected.

Protein content of the fractions was determined spectrophotometrically at 230 nm, and fractions were assayed for acid phosphatase and ATPase activity.

Cellulose acetate electrophoresis. Cellulose acetate strips were soaked in 0.04 M sodium acetate buffer, pH 5.4. Electrophoresis was run at 200 V potential difference for 30 min, after which Ponceau S staining was applied.

Results

Purification procedure

All the purification steps were carried out at 4° C.

Initial steps. The original solution containing the enzyme secreted by yeast protoplasts was first dialyzed 24 h against water, then an additional 24 h against 0.2 M sodium acetate buffer, pH 3.8. The volume of the enzyme solution was reduced to 20 ml by ultrafiltration. This operation resulted in removal of most proteins with molecular weights below 100 000. After readjusting pH to 5.5, the solution was further concentrated in a collodion bag, at reduced pressure.

Ion-exchange chromatography. The concentrated enzyme solution was run through the column of DEAE-Sephadex. Protein-containing material emerged in three portions (three peaks on Fig. 1). The first portion appeared during buffer elution and the other two during salt gradient elution. Pooled fractions, corresponding to each of these peaks, were associated with hydrolytic activity when tested with either *p*-nitrophenylphosphate (NPh-*P*) or ATP as substrates, and the ratio of these activities was the same for all peaks and was equivalent to that in the original solution.

The greater part of proteins (60%) occurred within the first peak. The frac-

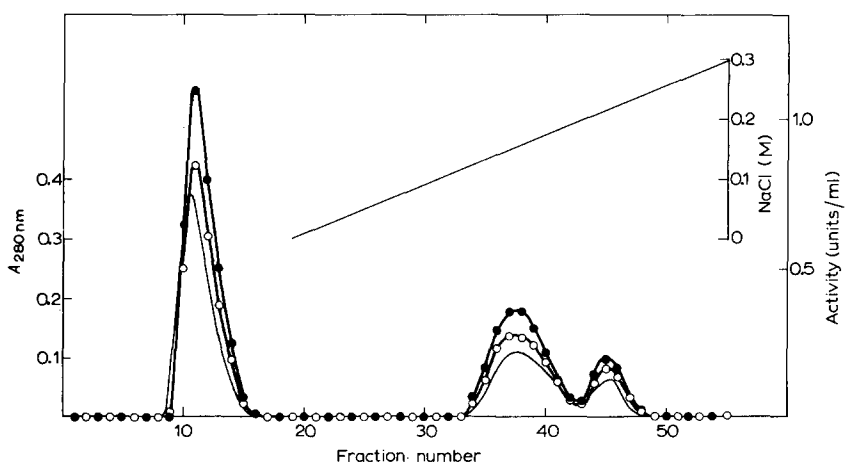

Fig. 1. DEAE-Sephadex A-25 column chromatography of the enzyme preparation secreted by protoplasts, after ultrafiltration (see text). The effluent was collected in 1-ml fractions at a rate of 1.5 ml/h. ———, absorbance at 280 nm; o———o, acid phosphatase; and •———•, ATPase activity.

tions of this peak were pooled, adjusted to pH 4.6, concentrated and taken to the next purification step. Material from the two other peaks was not further examined.

Gel filtration. A run through the Sephadex G-200 column gave a single, but asymmetrical peak. Its front fraction possessed lower activity than the other fractions. To eliminate inactive proteins, all the fractions were pooled, reduced in volume, and run through the Sepharose 4B column. This treatment removed an inactive protein of high molecular weight, leaving a purified preparation that gave a symmetrical elution profile on rerunning through the Sephadex G-200 column (Fig. 2).

The solution resulting after Sepharose 4B treatment (concentrated to 5 mg/ml of proteins) lost about 25% of its activity after 1 month's standing at 4° C.

Results of the stepwise purification are summarized in Table I. The overall effect was about 60-fold increase in both acid phosphatase and ATPase-specific activities, a steady ratio of activities being retained in all individual steps, and remaining at the level found in starting material.

Attempts to separate two enzyme activities

Isoelectric focussing. As shown in Fig. 3, a single, symmetrical peak was obtained by isoelectric focusing of the purified enzyme in the pH range from 3.5 to 5.5. This peak was endowed by both acid phosphatase and ATPase activities.

The estimated isoelectric point was about 4.6. A more exact determination would require an ampholyte with a narrower pH range.

Sucrose density gradient centrifugation. The results of sucrose density gradient centrifugation are shown in Fig. 4. Protein fractions, containing enzyme activities toward NPh-*P* and ATP, sedimented in identical patterns, maximum activity being always associated with the same fraction.

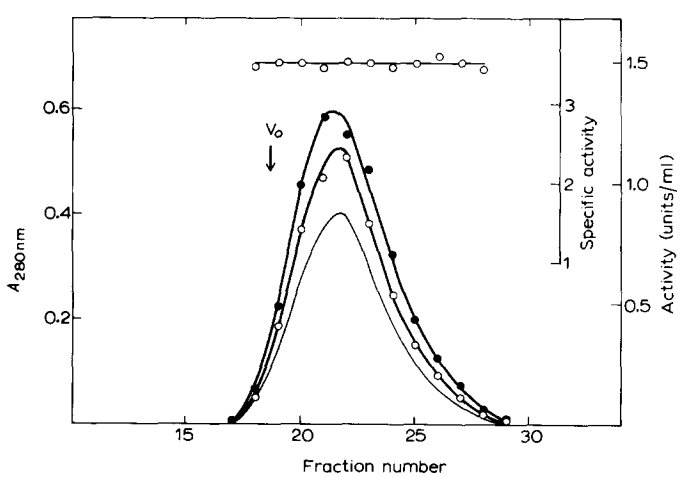

Fig. 2. Sephadex G-200 chromatography of the enzyme preparation, after Sepharose 4B. ———, proteins; o———o, acid phosphatase; •———•, ATPase activity. The specific activity of acid phosphatase in the peak fractions was 3.5 units/mg.

TABLE I

PURIFICATION OF ACID PHOSPHATASE SECRETED BY YEAST PROTOPLASTS

Purification step	Total protein (mg)	NPh-P as substrate			ATP as substrate			Specific activity ratio NPh-P / ATP
		Activity		Yield (%)	Activity		Yield (%)	
		Total (units)	Specific (units/mg)		Total (units)	Specific (units/mg)		
Secreted enzyme after dialysis	7200	440	0.061	100	960	0.133	100	0.46
Ultrafiltration	105	238	2.26	54	525	5	55	0.45
DEAE-Sephadex:								
Peak I	32	94	2.93	21.5	205	6.4	32	0.46
II	11	61	2.89	—	132	6.3	—	0.46
III	1.4	3.9	2.81	—	92	6.6	—	0.43
Sepharose 4B	21	76	3.58	17.5	170	8.1	18	0.44

Sedimentation constants, $s^{0.725}_{20,w}$, of purified enzyme were 16.2 S with respect to bovine liver catalase (11.3 S), and 16.1 S with respect to bovine serum albumin (4.6 S). A calculated value $s_{20,w}$ of 15.7 S was obtained on the basis of data reported by Martin and Ames [3], using a value of 0.69 cm^3/g (P. Boer, personal communication) for the partial specific volume of yeast acid phosphatase.

Properties of purified enzyme preparation

Electrophoresis. A single band was obtained on cellulose acetate strips by electrophoresis at pH 5.4.

Electrophoresis in polyacrylamide gel by Davis' method [4] was not informative, as the enzyme protein could not penetrate the gel, presumably because of its high molecular weight.

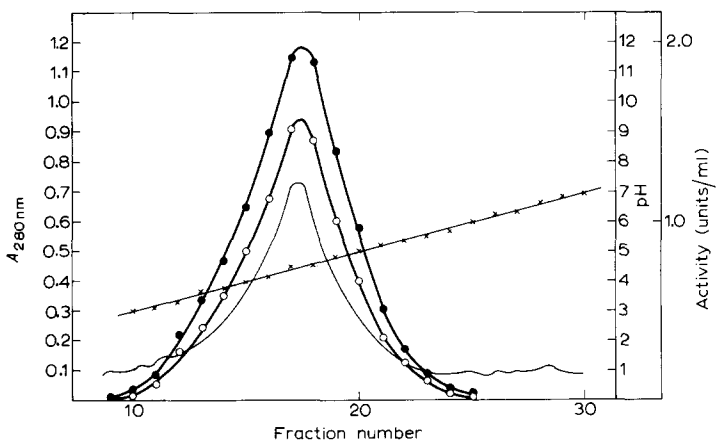

Fig. 3. Isoelectric focusing of the purified enzyme on a pH 3—10 sucrose linear gradient. ———, absorbance at 280 nm; o———o, acid phosphatase; •———•, ATPase activity; X———X, pH gradient.

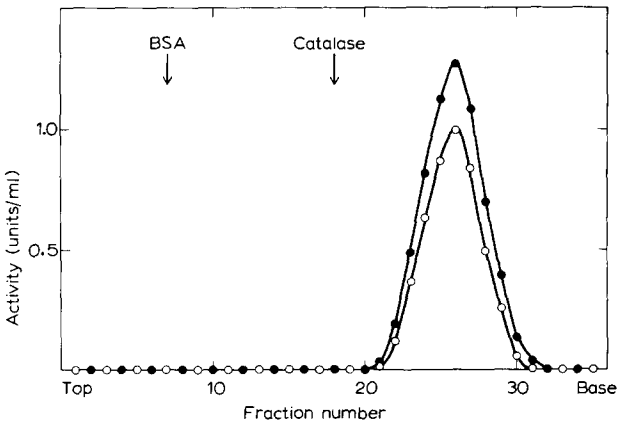

Fig. 4. Sucrose density gradient centrifugation of purified enzyme. Bovine liver catalase (11.3 S) and bovine serum albumin (4.6 S) were used as markers, which are indicated by arrows in the figure. For legend see Fig. 1.

Molecular weight. The molecular weight of purified enzyme was estimated by Sephadex G-200 gel filtration according to the method of Andrews [5]. A linear relationship was obtained between elution volume and log(molecular weight) with proteins of known molecular weight (Fig. 5A). By interpolation, the approximate molecular weight for acid phosphatase was 560 000.

Gel filtration data were also evaluated for estimating the diffusion coeffi-

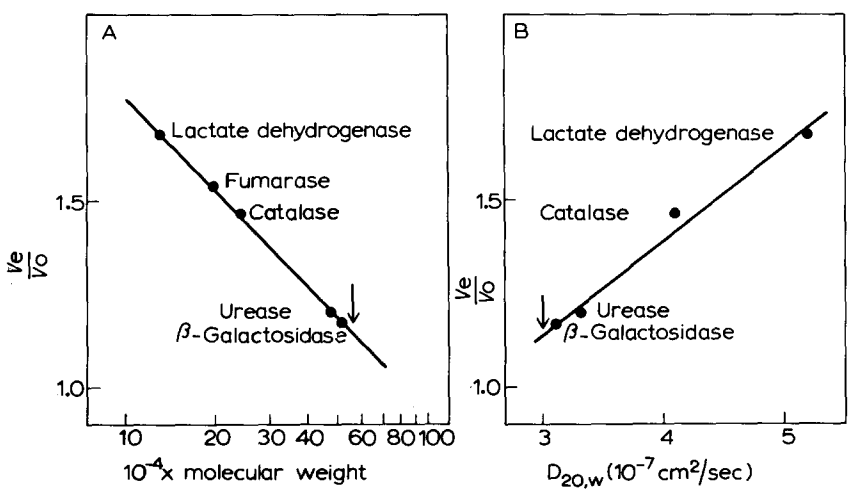

Fig. 5. Molecular weight (A) and diffusion coefficient (B) determinations for acid phosphatase by Sephadex G-200 gel filtration. The column was calibrated with proteins of known molecular weight and diffusion coefficient, $D_{20,w}$, (in parentheses): lactate dehydrogenase, rabbit muscle (130 000, 5.20), fumarase, pig heart, (194 000, —), catalase, bovine liver, (247 000, 4.10), urease, jack bean (483 000, 3.30), β-galactosidase, *Escherichia coli* (515 000, 3.12). Experimental V_e/V_0 values are plotted versus log(molecular weight) (A), and $D_{20,w}$ (B). The position of acid phosphatase is indicated by the arrows. Acid phosphatase molecular weight was estimated to be 560 000, and diffusion coefficient of $3.0 \cdot 10^{-7}$ cm^2/s was found.

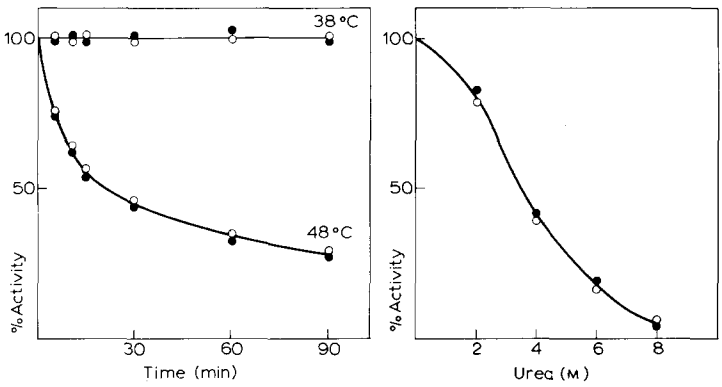

Fig. 6. Thermal inactivation of the purified enzyme. The sample solution was 1 ml and heated at the indicated temperature (38 and 48°C), and aliquots were removed at intervals to assay the enzyme activities. For symbols see Fig. 1.

Fig. 7. Effect of urea on purified enzyme. The standard reaction mixtures were incubated with various concentrations of urea. For symbols see Fig. 1.

cient. Graphical determination of the diffusion coefficient (Fig. 5B) gave $D_{20,w} = 3.00 \cdot 10^{-7}$ cm^2/s.

Molecular weight determination from sucrose density gradient centrifugation by the method of Martin and Ames [3], with bovine liver catalase, or bovine serum albumin as reference proteins, gave the values 405 000 and 428 000, respectively.

Calculations according to Siegel and Monty [6], $M = 6\pi\eta Nas/(1-\upsilon\rho)$, substituting the value 71.5 for Stokes' radius, calculated by means of the Stokes-Einstein equation, $a = kT/(6\pi\eta D)$, gave a molecular weight of 411 000.

Thermal inactivation. Heating at 38°C for 90 min had no effect on either ATPase or acid phosphatase activities, but at 48°C, during the same time, inactivation was observed, and these activities declined at the same rate (Fig. 6).

Urea denaturation. Acid phosphatase and ATPase activities were reduced to the same extent in the presence of increasing amounts of urea. The concentra-

TABLE II

EFFECT OF NH_4^+ ON ACID PHOSPHATASE AND ATPase ACTIVITIES

The enzyme activities were determined as cited in Materials and Methods. The results are expressed as percentage of control.

NH_4^+ concentration (mM)	Residual activities (%)	
	Acid phosphatase	ATPase
0	100	100
2.8	80	68
28	40	13
280	9	0

TABLE III

EFFECT OF SULFHYDRYL REAGENTS ON ACID PHOSPHATASE AND ATPase ACTIVITIES

The enzyme activities were determined as cited in Materials and Methods. The results are expressed as percentage of control measured in the absence of the respective reagent.

Reagent (M)	Remaining activities (%)	
	Acid phosphatase	ATPase
None	100	100
$HgCl_2$ (10^{-4})	6	3
Phenylmercuric acetate (10^{-3})	67	51
Iodoacetate (10^{-3})	90	80

tion of urea exerting 50% inhibition was 3.5 M with regard to both activities (Fig. 7).

Effect of the salts of Markham-mannitol medium on enzyme activities. The two activities behaved similarly in response to the presence of the salts of Markham-mannitol medium. KCl, $CaCl_2$, $MgSO_4$, $MnSO_4$, $ZnSO_4$ and $FeCl_3$ added individually, to standard reaction mixture, in concentrations as in the Markham-mannitol medium, had no effect on either ATPase or acid phosphatase activities. Citrate ion (1.5 mM) enhanced both acid phosphatase (25%) and ATPase activity (30%), whereas NH_4^+ inhibited both activities (Table II).

Sulfhydryl reagents. These compounds inhibited ATPase and acid phosphatase activity to nearly the same extent, but the overall inhibitory effect, at these concentrations of the reagents, was rather low (Table III).

Discussion

The purification of protoplast-secreted acid phosphatase was carried out and an attempt was made to separate enzyme activities catalyzing the hydrolysis of NPh-*P* and ATP, respectively, which are always observed in association in crude material.

A purification of yeast acid phosphatase from intact cells has been previously carried out by Boer and Steyn-Parvé [7].

In the present work a different starting material, an enzyme preparation secreted by yeast protoplasts, was used.

DEAE-Sephadex ion-exchange chromatography, yielded the three well-resolved peaks, as it did in experiments reported by Boer et al. [8]. Fractions corresponding to each of these peaks still contained acid phosphatase and ATPase activities, and that, in the same ratio as in the initial preparation. At this moment we shall make no attempt to decide whether the three peaks resulting from ion-exchange chromatography represent different isozymes, or just one type of glycoprotein with different number of phosphate groups per molecule.

Results presented in this paper suggest that the catalytic activities toward NPh-*P* and ATP hydrolysis, reside in the same protein molecule. This statement is based on the following observations: (1) The two activities behave in the

same way when subjected to ion-exchange with DEAE-Sephadex A-25, and to gel filtration. (2) The acid phosphatase/ATPase ratio remains unchanged after each purification step. (3) The two activities cannot be separated by sucrose density gradient centrifugation, isoelectric focusing experiments and cellulose acetate electrophoresis. (4) The rate of decline of both activities at elevated temperature as well as in the presence of urea was the same.

Sulfhydryl reagents inhibited both activities similarly, but the inhibition was rather low, even at relatively high inhibitor concentrations. Thus, sulfhydryl groups probably do not constitute a part of the catalytic site.

The purified enzyme, as obtained by the procedure applied in this work, appeared to be a fairly large molecule. Its molecular weight determined by graphical interpolation of gel filtration data is 560 000. The figure exceeds the values calculated on the basis of sucrose density gradient centrifugation data. The high value could be explained by recalling that acid phosphatase is a glycoprotein [7] which usually show an anomalous behaviour in gel filtration [5]. On the other hand, the relationships of elution data of gel filtration and diffusion coefficient of glycoproteins are mostly quite normal and the later can be correctly estimated [5]. We used, therefore, the diffusion coefficient, obtained by gel filtration, in the calculations of molecular weight according to Siegel and Monty [6]. Thus obtained molecular weight (411 000) agrees fairly well with the value calculated by the method of Martin and Ames [3] (405 000 and 428 000).

These figures, however, still considerably exceed the molecular weight reported by Van Rijn et al. [9] (290 000) for yeast acid phosphatase, although our value for $s_{20,w}$ was very close to that reported by Boer and Steyn-Parvé [7] (15 S). The difference might be due to different methods of determination, or we might be dealing here with a different molecule, with respect to the state of aggregation.

Acknowledgments

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