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PURIFICATION AND EVIDENCE FOR HETEROGENEITY OF ACID PHOSPHATASE FROM SACCHAROMYCES CEREVISIAE

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SUMMARY: The protoplast-secreted acid phosphatase of yeast Saccharomyces cerevisiae was purified about 60 fold by ultrafiltration, gel filtration and chromatography on DEAE-Sephadex A-25. It was established that the enzyme is free of inactive proteins as well as polysaccharides and contains 48 % of neutral sugars. The failure to separate the protein from the carbohydrates by several procedures indicates that the carbohydrate part is covalently linked to the protein. A pronounced heterogeneity of the enzyme with respect to charge as well as to molecular weight was found. The data obtained by gel filtration indicated enzyme heterogeneity in respect to carbohydrate content.

INTRODUCTION

Acid phosphatase (EC 3.1.3.2.) is an extracellular enzyme forming part of the yeast cell wall matrix (1, 2, 3, 4).

Under appropriate conditions, yeast protoplasts are able to secrete acid phosphatase, and the released enzyme is identical to the enzyme located in the intact yeast cell wall (5).

The protoplast-secreted enzyme resolves into several fractions during ion-exchange chromatography indicating the existence of several molecular forms (6, 7).

In the present paper we describe the purification of the protoplast-secreted enzyme giving rise to an enzyme preparation of high degree of purity. Strong evidence is presented for heterogeneity of the enzyme with respect to charge and molecular weight.

MATERIALS AND METHODS

Determination of proteins. Proteins were determined by the method of Lowry et al. (8).

Carbohydrate determination. Neutral sugars were determined by the orcinol-H_2SO_4 method, (9), and by the phenol-H_2SO_4 method (10).

Enzyme assay. Acid phosphatase activity was determined with p-nitrophenyl-phosphate as substrate. One unit of the enzyme activity was defined as the amount of the enzyme that hydrolyses 1 μmol of substrate per minute (0.1 M acetate buffer, pH 3.8, 30°C).

α-D-Mannosidase activity was determined with p-nitrophenyl- α-D-mannopyranoside as substrate in 0.1 M acetate buffer, pH 5.0.

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Precipitation of the enzyme with Concanavalin A. Enzyme solution (~ 0.5 mg protein) in 0.1 M acetate buffer, pH 4.90, containing 10^{-3} M $MgCl_2$, 10^{-3} M $MnCl_2$ and 1 M NaCl, was mixed with four different concentrations of Con A. After incubation with mixing for 12 h at 30°C, the solution was clarified by centrifugation and the carbohydrate content and enzyme activity in the supernatant were measured. A control sample without Con A was treated in the same way.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed in 5 % gel. The buffers used were: 0.01 M imidazole-HCl buffer, pH 6.4 and 7.4, and 0.01 M glycin-HCl buffer, pH 3.0. For protein detection, gels were stained with Coomassie Brilliant Blue R-250. Phosphatase activity was localized by a diazo coupling method using 0.1 M acetate buffer, pH 3.8, containing 2 mg/ml disodium α-naphthylphosphate and 5 mg/ml Fast Red B. The detection of carbohydrates was performed by periodic acid-Schiff reaction (11). Gels were scanned in Densitometer DD2, Kipp & Zonen Holland, using filter C (500-550 nm). SDS-electrophoresis was performed in 0.1 % sodium dodecyl sulphate using 0.1 M Na-phosphate buffer, pH 7.2 (12).

Isoelectric focusing. Isoelectric focusing was carried out at 4°C in 110 ml column, (LKB Produkter AB, Stockholm, Sweden). Ampholytes (pH 3-6) were used at 1 % concentration. Analytical gel electrofocusing (pH 3-6) of the enzyme was performed at 15°C on cylindrical 5 % gels as described by Wrigley (13).

Enzyme purification. The yeast S. cerevisiae (strain 367, Technological Faculty Collection, Zagreb) was cultured aerobically in phosphate-poor medium (14), in glass laboratory fermentor for 10 h at 30°C. Protoplasts were prepared from yeast cells (5, 15) and the crude enzyme preparation was obtained by secretion from protoplasts incubated in Markham-mannitol medium (5).

The crude enzyme solution (~ 30 liters) was dialyzed against 0.1 M-acetate buffer, pH 3.8 and concentrated by ultrafiltration using Modul 36 LAB (De Danske Sukkerfabriker, Denmark) to 3 liters and then, by using an ultrafiltration cell (Model 202, Amicon Corp., Holland) provided with XM-300 A membrane (mol.wt. cut-off 300 000), to 10 ml. 150 ml 0.1 M Acetate buffer, pH 4.6 was added and the enzyme solution was concentrated again to 10 ml using the same membrane. This last step was repeated.

The concentrated enzyme solution was filtered through a column (2.5 cm x 95 cm) of Ultrogel AcA 22 equilibrated with 0.1 M acetate-buffer, pH 4.6. The column was eluted at a flow rate of 12 ml/h. By this procedure the enzyme was separated from an inactive high molecular weight material eluted in void volume (not shown).

Concentrated enzyme solution was then applied to a column (2.5 cm x 45 cm) of DEAE-Sephadex A-25, which has been equilibrated with 0.025 M acetate buffer, pH 4.9. The column was washed with this buffer until the A_{280} of the eluate was 0.05, eluted with 800 ml linear gradient of 0.025 M acetate buffer, pH 4.9-3.7, continuing with 0.025 M acetate buffer, pH 3.7 until the A_{280} of the eluate dropped to zero. The column was then eluted by gradual increase of ionic strength of acetate buffer, pH 3.7 (0.025 M to 0.5 M). Flow rate of 90 ml/h was applied at room temperature (22°C).

RESULTS AND DISCUSSION

Purification procedure and the results of the protein, carbohydrate and enzyme activity determination are presented in Table 1. Fractions DEAE I and III (Fig. 1.), containing only 5 % and 2% of total activity, respectively, were contaminated with inactive proteins and glycoproteins and were not further purified. Fraction DEAE II which con-

Table 1. Purification of acid phosphatase from Saccharomyces cerevisiae. For details see the text.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Carbohydrates (%)
Crude enzyme preparation (protoplast secreted enzyme)	14 000	16 000	1.14	100	82
Ultrafiltration	430	13 000	30.02	81	86
Ultrogel AcA 22 chromatography	270	12 000	43.50	75	81
DEAE-Sephadex A-25 chromatography					
fraction I	26	405	15.60	-	93
fraction II	130	9 350	72.00	58.5	48
fraction III	6	180	30	-	57

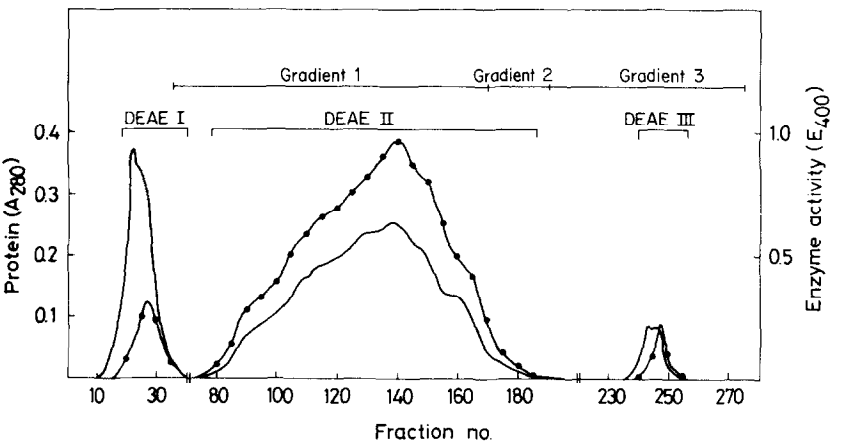
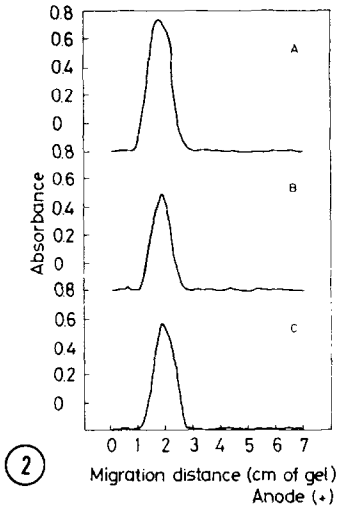


Fig. 1. DEAE-Sephadex A-25 chromatography of acid phosphatase:
Gradient 1: 400 ml of 0.025 M acetate buffer, pH 4.9 + 400 ml of 0.025 M acetate buffer, pH 3.7.
Gradient 2: 120 ml 0.025 M acetate buffer, pH 3.7
Gradient 3: 250 ml 0.025 M acetate buffer, pH 3,7 + 250 ml od 0.50 M acetate buffer, pH 3.7.
——— protein, o——o enzyme activity

tained more than 90 % of total activity and displayed the highest specific activity was heterogeneous within itself. No increase in specific activity was observed after rechromatography of this fraction on the same column and the protein/carbohydrate ratio was found to be unchanged. Specific activity and the carbohydrate content (48 %) of the purified enzyme is very similar to the values reported for acid phosphatase from intact cells of S. cerevisiae (16).



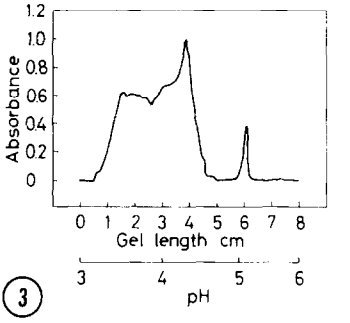


Fig. 2. Electrophoretic analysis of acid phosphatase (fraction DEAE II), at pH 6.4 The gels were stained for enzyme activity, (A), carbohydrates, (B), protein, (C), and scanned as described in Materials and Methods.

Fig. 3. Densitometric scan of acid phosphatase subjected to isoelectric focusing in 5 % polyacrylamide gel, pH range 3-6. Gels were stained for enzyme activity as described in Materials and Methods.
Gel isoelectric focusing was performed with 5-10 μg of protein under conditions described in the text. For pH gradient determination, unstained gel, run simultaneously, was cut in 20 sections which were eluted with 2 ml of water and the pH of the extracts was measured.

Purified acid phosphatase (fraction DEAE II) was subjected to electrophoresis at three pH values: 3.0; 6.4 and 7.4. In each case a single, but rather diffuse band was detected after staining for proteins, carbohydrates and enzyme activity (Fig. 2). Similar pattern was obtained by electrophoresis of acid phosphatase from Candida albicans (17). By SDS-electrophoresis a rather diffuse but still single band was obtained after staining for protein and carbohydrate (not shown), confirming purity, as well as heterogeneity of the enzyme.

Isoelectric focusing in sucrose density gradient (pH 3-6) gave one main peak of enzyme activity, having pI 4.3, as well as several smaller peaks isoelectric in the pH range 3.3 - 5.3. By analytical isoelectric focusing in polyacrylamide gels, which offers faster and more convenient analyses (18) the enzyme was resolved into multiple components. Main fraction was isoelectric at pH 4.25 (Fig. 3). Prolonged experiments (further 4 hrs) gave the same pattern. Essentially the same banding was obtained when the samples were introduced by loading on top of preformed gels or by mixing throughout the gel solution before polymerization. From this, it is unlikely that the heterogeneity is a consequence of artifact formation. Similar heterogeneity was also found by isoelectric focusing experiments for yeast invertase (19, 20, 21).

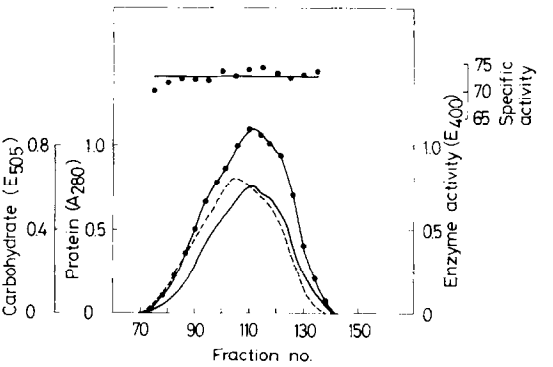


Fig.4. Gel filtration of acid phosphatase on Ultrogel AcA 22 (see text).
——— protein, ------ carbohydrates, o———o enzyme activity

Gel filtration of the enzyme on Ultrogel AcA 22 gave a single, but broad peak with constant specific activity through the fractions. The carbohydrate content after gel filtration measured through the fractions, was 55 % in the first fractions (fractions 75 to 85), declining gradually to 43 % in the last fractions (fractions 125 to 135) (Fig.4). This indicated that the enzyme preparation consists of a mixture of molecules with varying amounts of carbohydrate.

The experiments described above indicated charge and molecular size heterogeneity of acid phosphatase. The failure of better resolution of the enzyme fractions by the procedures used, suggests that the enzyme preparation consists of a whole range of molecules which slightly differ one from another.

The covalent association between carbohydrate and enzyme protein was indicated by failure to separate the carbohydrate from protein and was further confirmed by precipitation of the enzyme with Con A. Results of this experiment, presented in Table 2, show that the enzyme protein precipitated together with the carbohydrate present in the solution.

Table 2. Precipitation of acid phosphatase with Concanavalin A. Experiment was performed as described in Materials and Methods. Carbohydrate content and enzyme activity of the control sample, incubated under the same conditions in the absence of Con A, are expressed as 100 %.

Con A added (mg)	Carbohydrate (%)	Enzyme activity (%)
0	100	100
0.2	80	78
0.4	62	61
0.8	40	38
1.2	32	30

Crude enzyme preparation was tested for α-mannosidase activity which if present, might be a cause of heterogeneity of the enzyme. Since no α-mannosidase activity was detected in the enzyme preparation, the observed heterogeneity was not the consequence of a possible degradation by glycosidase.

The work dealing with composition and structure of the carbohydrate part of the enzyme is in progress and the results obtained confirmed that heterogeneity resides in the carbohydrate part of the enzyme molecule.

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