Physicochemical and Kinetic Properties of Acid Phosphatase from Saccharomyces cerevisiae*

Slobodan Barbarić, Branko Kozulić, Blanka Ries, and Pavao Mildner
From the Laboratory of Biochemistry, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeja 6, 41000 Zagreb, Yugoslavia

Acid phosphatase from yeast Saccharomyces cerevisiae was purified, and its physicochemical and kinetic properties were investigated. The biosynthesis of invertase, the most extensively studied yeast extracellular enzyme, is regulated by the amount of glucose present in the culture medium (1, 2), while inorganic phosphate present in the medium is a regulatory factor in the biosynthesis of acid phosphatase (3, 4). Besides the repressible acid phosphatase, yeast contains a low level of a constitutive enzyme (5, 6).

The diffusion constant has been found to be $d_{20, w} = 3.9 \times 10^{-7}$ cm² s⁻¹, and the calculated partial specific volume was $\bar{v} = 0.663$ cm³/g. From these data, a molecular weight of 252,000 was calculated. Electrophoresis on gel slabs, with a linear concentration gradient of polyacrylamide (4–30%), showed size heterogeneity of the native enzyme preparation and indicated an apparent molecular weight in the range of 170,000 to 360,000.

In the presence of sodium dodecyl sulfate, the molecular weight was in the range of 82,000 to 165,000, indicating dimeric structure of the native enzyme, which was confirmed by cross-linking experiments. Isoelectric focusing demonstrated charge heterogeneity of enzyme preparation.

From CD spectrum it was calculated that the enzyme contains about 29% of $\alpha$-helical structure. Excitation at 278 nm gave an emission fluorescence spectrum with a maximum at 340 nm.

Amino acid analysis revealed a high content of aspartic acid, serine, and threonine. Glycine is found as the $NH_2$-terminal amino acid.

Initial velocity dependence on substrate concentration, as well as on pH, and thermostability studies indicated the presence of at least two enzyme forms in the preparation.

The yeast cell has a number of hydrolytic enzymes localized externally to the plasma membrane retained by the cell wall. The level of some of these enzymes depends on yeast growth conditions. The biosynthesis of invertase, the most extensively studied yeast extracellular enzyme, is regulated by the amount of glucose present in the culture medium (1, 2), while inorganic phosphate present in the medium is a regulatory factor in the biosynthesis of acid phosphatase (3, 4). Besides the repressible acid phosphatase, yeast contains a low level of a constitutive enzyme (5, 6).

Represible acid phosphatase was purified from crude extract, obtained by disruption of intact yeast cells (7) as well as by protoplast secretion (8). The enzyme is a glycoprotein with about 50% carbohydrates, and for the protoplast-secreted enzyme, it was established that carbohydrate chains are $N$-glycosidically linked to protein and consist of mannose and $N$-acetylglucosamine (9). A pronounced heterogeneity of acid phosphatase, according to charge and molecular weight, was demonstrated (8). It was suggested that the enzyme has a homogenous protein part and that heterogeneity resides in the carbohydrate part, as a consequence of different length of carbohydrate chains linked to protein (9).

Biosynthesis of the enzyme and its regulation by inorganic phosphate concentration in the growth medium (10–13), secretion by protoplasts, as well as some kinetic properties of acid phosphatase were extensively studied (14–16); but in contrast to invertase, detailed physicochemical characterization has not yet been performed.

The present paper deals with physicochemical properties of the enzyme such as amino acid composition, molecular weight of the native and dissociated enzyme, spectroscopic data, and kinetic properties.

EXPERIMENTAL PROCEDURES

RESULTS

Purity of Enzyme Preparation—Purified acid phosphatase was subjected to electrophoresis, and a single diffuse band was detected after staining for proteins and for enzyme activity (Fig. 1), showing that the enzyme is free of inactive proteins. Prolonged electrophoresis brought about substantial loss of the enzyme activity, because of its instability at the operating pH (see below); so the staining for enzyme activity was impossible; but even by such prolonged electrophoresis, resolution of the diffuse band into separate protein zones was not achieved. Purity of the enzyme was further demonstrated by gel filtration experiments, where a constant specific activity through collected fractions was found and by a single schlieren peak obtained in the ultracentrifuge (not shown). Gel electrophoresis of the enzyme on gel slabs, with a linear gradient of polyacrylamide concentration (where proteins are resolved on the basis of their size), further confirmed the purity of the enzyme since only a single, diffuse band was obtained (see below, Fig. 3). By SDS-gel electrophoresis, only one wide band was also obtained (see below, Fig. 4).

Molecular Weight and Subunit Composition—Sedimentation coefficients were determined at different enzyme concentrations (1–8 mg/ml). A typical sedimentation velocity pattern

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1 Portions of this paper (including "Experimental Procedures" and Figs. 1 and 8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 83 M-1918, cite the authors, and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
obtained by absorption measurements is shown in Fig. 2. By linear extrapolation to infinite dilution, \(s_{20,w}\) was determined from three parallel experiments, and the mean value of 13.6 S was taken for molecular weight calculation. The diffusion constant was also determined in the ultracentrifuge at several protein concentrations, and it was calculated to be \(3.9 \times 10^{-14}\) cm s\(^{-1}\) at infinite dilution. Partial specific volume calculated from chemical composition of the enzyme was 0.663 cm\(^3\)/g. Using the Svedberg equation, an \(M_r = 252,000\) was calculated from the data given above.

Molecular weight of the native enzyme was determined also by electrophoresis on gel slabs with a linear concentration gradient of polyacrylamide (4–30%), by comparison with proteins of known molecular weight. This method clearly showed heterogeneity of the enzyme preparation and indicated molecular weights in the range of 170,000 to 360,000 (Fig. 3).

Subunit molecular weight was estimated by gel electrophoresis on a gradient gel slab in the presence of sodium dodecyl sulfate. Only one wide band was observed, showing again heterogeneity of the enzyme (Fig. 4). Molecular weight was in the range of 82,000 to 165,000, indicating that the native enzyme is a dimer. Dissociation of the enzyme into subunits was obtained without treatment with 2-mercaptoethanol, showing that a disulfide bridge(s) is not included in association of the subunits. Evidence for the number of subunits was further sought by cross-linking of the enzyme subunits with glutardialdehyde. As shown by SDS-electrophoresis (Fig. 5), intramolecular cross-linking with glutardialdehyde yielded only one band with a molecular weight around 250,000, confirming that the native enzyme is a dimer.

Molecular Shape—Molecular weight and molecular radius are related to the frictional ratio by the equation of Onsley (25). Molecular radius was calculated using the equation of Siegel and Monty (26). Substitution of the value of 252,000 for the molecular weight and the value of 13.6 S for the sedimentation coefficient gave a molecular radius of 5.5 nm. Using this value, the frictional ratio was estimated to be 1.36.

Isoelectric Focusing—Results of isoelectric focusing of the enzyme preparation on gel plates are shown in Fig. 6. A diffuse band, in which some distinct sharp stripes can be seen, was obtained in the region which corresponds to pH from 4.1 to 4.7, determined on the basis of known pI of standard proteins focused in the same experiment.

Amino Acid Analysis—Amino acid composition of acid phosphatase is presented in Table I. The number of residues was calculated from the average of duplicate 22- and 72-h hydrolysates with the following exceptions. The values for valine and isoleucine were obtained from duplicate 72-h hydrolysates, whereas those for threonine and serine were calculated by extrapolation to zero time of hydrolysis. Tryptophan and tyrosine contents were determined spectrophotometrically according to Edelhoch (27). Large amounts of aspartic and glutamic acid, as well as threonine and serine, were detected. Glucosamine content was determined, too, and 32 mol were found. The number of sulphydryl groups in the enzyme was determined by the 5,5'-dithiobis(2-nitrobenzoic) method, and the results indicated that there is less than 1 mol of sulphydryl groups/mol of native enzyme. In the presence of 1% SDS, 1.3 mol/mol were determined; whereas in 8 M urea solution plus 1% SDS, nearly 2 mol of sulphydryl groups were found. NH\(_2\)-terminal amino acid was determined; and only one major product was found, and it co-chromatographed with 5-dimethylaminonaphthalene-1-sulfonyl glycine.

Spectral Properties—The ultraviolet absorption spectrum of the enzyme showed an absorption maximum at 278 nm and a small shoulder at 290 nm, indicating exposed tryptophan residues. The 280/360 nm absorbance ratio is 1.81, showing that the enzyme preparation is free of nucleic acids. The absorption coefficient at 278 nm (\(A_{278}^{278}\)) is 1.43 determined on the basis of dry weight (protein dry weight is taken as 50% of total dry weight). On the basis of \(M_r = 126,000\) for the protein part, the molar absorption coefficient is \(1.8 \times 10^4\) M\(^{-1}\) cm\(^{-1}\).

The fluorescence spectrum of acid phosphatase was also recorded. On excitation at 278 nm, the emission optimum was at 340 nm, which is characteristic for tryptophan fluorescence emission. Tyrosine did not contribute significantly to the emission spectrum since no shoulder at 310 nm was observed.

In Fig. 7, circular dichroism spectra of acid phosphatase in the near- and far-ultraviolet region are shown. In the far-ultraviolet region, the enzyme exhibits a negative CD band centered at 212 nm with molecular ellipticities \(\theta\) of about \(-11,000\) degrees \(\times\) cm\(^2\) \(\times\) dmol\(^{-1}\). Secondary structure of the enzyme was calculated using computer analysis based on the method of Chen et al. (28). The \(\alpha\)-helix content was estimated to be 29%, and the content of \(\beta\)-structure was 37%.

Stability and Kinetic Properties—Acid phosphatase is stable only in a very narrow pH range, from 3.0 to 5.0, and undergoes denaturation below and above these values. The purified enzyme is very thermolabile, too. During 15 min of incubation at 50 °C, enzyme activity decreased by one-half, and a nonlinear relationship between log (per cent activity) and time was
Properties of Yeast Acid Phosphatase

FIG. 3. Electrophoresis of acid phosphatase on gel slab with a linear concentration gradient of polyacrylamide (4–30%), performed as described under "Experimental Procedures". Lanes 1 and 3, 25 and 50 μg of acid phosphatase, respectively; lane 2, standard proteins: thyroglobulin (Mr = 669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000).

FIG. 4. SDS electrophoresis of acid phosphatase on gel slab with a linear concentration gradient of polyacrylamide (4–30%), performed as described under "Experimental Procedures." Lane 2, 50 μg of acid phosphatase; Lane 1, standard proteins: ferritin (Mr = 220,000), bovine serum albumin (67,000), catalase (60,000), and lactate dehydrogenase (36,000); lane 3, standard proteins: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

FIG. 5. Cross-linking of acid phosphatase with glutardialdehyde. SDS electrophoresis of treated and untreated acid phosphatase on gel slabs with linear concentration of polyacrylamide (4–30%). Lane 1, untreated enzyme; lanes 2, 3, and 4, the enzyme treated with 1% glutardialdehyde; concentration of protein during treatment was 30 μg/ml (lanes 2 and 4) and 500 μg/ml (lane 3); lane 5, standard proteins: as in Fig. 4, lane 1.

FIG. 6. Isoelectric focusing of acid phosphatase on Ampholine-polyacrylamide gel plate, pH range 3.5–9.5. Lane 2, acid phosphatase; lane 1, standard proteins.

Observed (not shown). In order to find which conformational changes are responsible for the loss of the enzyme activity, CD spectra were recorded at conditions where enzyme activity was completely and irreversibly lost, i.e. 60 °C or pH 10. Irreversible side chain perturbations were noticed (near-UV spectrum), while overall protein conformation (far-UV spectrum) did not change at all (Fig. 7).

Fig. 8 shows the effect of a wide range of p-nitrophenyl phosphate concentrations on enzymatic activity of acid phosphatase. A biphasic curve was obtained in the Lineweaver-Burk plot, from which two limiting Km values could be esti-
FIG. 7. Circular dichroism spectra of acid phosphatase. ——, 0.1 M NaOAc buffer, pH 4.6; —, 0.1 M glycine/NaOH buffer, pH 10.0, or 0.1 M NaOAc buffer, pH 4.6, recorded at 60 °C.

TABLE 1

<table>
<thead>
<tr>
<th>Amino acid composition of acid phosphatase</th>
<th>Residues calculated for M, = 252,000 (60% carbohydrate) (nearest integer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>172</td>
</tr>
<tr>
<td>Threonine</td>
<td>111</td>
</tr>
<tr>
<td>Serine</td>
<td>92</td>
</tr>
<tr>
<td>Glutamic acid</td>
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<td>Proline</td>
<td>34</td>
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<td>Glycine</td>
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<td>Cysteine</td>
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<td>Tryptophan</td>
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<td>Methionine</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
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<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Histidine</td>
<td>18</td>
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<tr>
<td>Arginine</td>
<td>26</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>26</td>
</tr>
<tr>
<td>Ammonia</td>
<td>200</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>32</td>
</tr>
</tbody>
</table>

TABLE 1 estimated: about 0.28 x 10^{-3} M at low substrate concentration and about 3.0 x 10^{-3} M at high substrate concentration. Besides that characteristic, the enzyme exhibits a substrate inhibition effect (see Fig. 8, inset).

Optimum pH for acid phosphatase activity lies between pH 3.0 and 4.5, with two poorly resolved maxima: one between 3.2 and 3.6 and another between 4.1 and 4.4 (not shown).

DISCUSSION

We have purified acid phosphatase from intact cells of Saccharomyces cerevisiae, and the pure enzyme contained about 50% carbohydrates (26% N-acetylglucosamine and the rest is mannose) and about 50% protein, by total weight, which is practically the same as that determined for the protoplast-secreted enzyme (8). By different electrophoretic analyses of the purified enzyme, the presence of a single enzymatically active band was demonstrated, showing that the enzyme is free of inactive proteins. The same experiments indicated heterogeneity of the enzyme. Electrophoretic heterogeneity of acid phosphatase was observed by other authors, too (29). Sedimentation velocity experiments did not give clear evidence about heterogeneity of the enzyme; but during diffusion measurements, non-gaussian shape of the schlieren peak was noticed, indicating heterogeneity of the enzyme. Electrophoresis on gradient gel slabs clearly showed size heterogeneity of the native enzyme, which was further demonstrated by SDS electrophoresis. A wide range of molecular weights found for acid phosphatase subunits is in agreement with our previous results obtained with the protoplast-secreted enzyme, where enzyme subunits were resolved by gel filtration in the presence of SDS, in fractions varying in mannose content from 38 to 70% (8). After SDS electrophoresis on slabs with a gradient of polyacrylamide concentrations, an anomalous behavior of glycoproteins on SDS electrophoresis (reduced charge/mass ratio relative to unglycosylated proteins which diminishes their migration rate) was mostly abolished (31), and the obtained values for molecular weights could be considered legitimate.

We have found that deglycosylated subunits have molecular weights around 68,000, in agreement with the findings of other authors (32, 33). From this result, the calculated molecular weight for the molecule containing in average 50.5% sugars (as found particularly for this preparation) is 243,000 for the dimer and 121,500 for subunits, which is very close to the mean molecular weights for the native and dissociated enzyme experimentally obtained in this work. A somewhat higher value (290,000), obtained by ultracentrifugation, was previously reported for the native acid phosphatase from S. cerevisiae (34). It was found that acid phosphatase from Schizosaccharomyces pombe exists as a dimer-tetramer dissociating nonequiliabrating system with a dimer M, = 180,000 (35). For acid phosphatase from Candida albicans, it was reported, that the native enzyme is a monomer with mean M, = 131,000 (estimated by SDS electrophoresis), a value similar to that which we obtained for subunits of our enzyme (36). In contrast to this difference among the reported values for molecular weights of acid phosphatase from different yeast genera, for invertase from S. cerevisiae, similar size heterogeneity was demonstrated by SDS electrophoresis and subunit molecular weight was found to be in nearly the same range (90,000 to 160,000 (37)) as we determined for acid phosphatase preparations. The molecular weight of the native dimeric enzyme is also very similar to the value found for our acid phosphatase preparation (38, 39).

S. Barbaric, V. Mrša, B. Ries, and P. Mildner, manuscript in preparation.
Besides size heterogeneity, a charge heterogeneity of the enzyme was demonstrated by isoelectric focusing, which is in agreement with our previous finding for the protoplast-secreted enzyme (8). Different degrees of phosphorylation, which was suggested as a cause of charge heterogeneity in invertase (40), can be only partially responsible for acid phosphatase heterogeneity because we found less than 1 mol of phosphate/mol of enzyme. Thus, the existence of different protein chains in an acid phosphatase preparation, indicated by deglycosylation experiments and reported by other authors (32, 33), cannot be excluded. However, in our experiments, only one NH_2-terminal amino acid was detected; but this could be explained by an identical NH_2-terminus part. In agreement with the above findings, kinetic data presented in this paper also suggest the possible presence of at least two enzyme forms in the preparation which have different values, pH optima, and thermostability. The same behavior was already reported for the protoplast-secreted enzyme preparation (15).

Data presented here show that acid phosphatase, despite a high amount of attached sugar, is a relatively unstable enzyme, as also noticed by other authors for the S. cerevisiae enzyme (29) and the enzyme from S. pombe (41). It is interesting to note a relatively high amount of α-helical structure in acid phosphatase in comparison with other investigated glycoproteins which are usually either devoid of α-helical conformation or have only little of it (42). It was demonstrated, by deglycosylation of the enzyme, that carbohydrate chains did not contribute to the CD spectrum of acid phosphatase, and the same was found for invertase (43).

Comparing our results of amino acid composition with the amino acid composition of acid phosphatase from S. pombe (41), some similarity could be observed; this is less obvious in the case of acid phosphatase from Rhodotorula rubra (44); but, interestingly, with invertase from S. cerevisiae (38), much higher similarity was noticed. Two sulphydryl groups/mol of acid phosphatase were determined under denaturing conditions, and a similar value was found for invertase (38). In contrast, the absence of free thiol groups in acid phosphatase from S. pombe was reported (41).

We found about 32 molecules of N-acetylglucosamine/enzyme molecule. Taking into account that two glucosamine molecules are included in a N-glycosidic link (45), one can assume that there are 16 carbohydrate chains/enzyme molecule, or 8/subunit. For invertase, a similar value of 18 chains or 9/subunit was reported (37). Generally, a high similarity in chemical composition, as well as in size and quaternary structure, between acid phosphatase and invertase from S. cerevisiae could be observed. In addition to similarity in the protein part, acid phosphatase and invertase have almost the same carbohydrate parts (45, 46), and it appears that acid phosphatase, like invertase (37), is a dimer consisting of two subunits with protein chains of molecular weight about 60,000. This similarity between two different enzymes could be a result of general requirements which need to be fulfilled for biosynthesis, secretion, and/or control of yeast repressible extracellular glycoenzymes.

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REFERENCES


SUPPORTING MATERIAL

**Title:** Posthinoic and kinetic properties of acid phosphatase from yeast cultures

**Authors:** Slobodan Barbasid, Blanka Ralko, and Ivoj Mijaden

**EXPERIMENTAL PROCEDURES**

**Chemicals and materials:** DIAL – p-Nitrophenylphosphate, pH 4.0, buffer (0.1 M sodium acetate, 0.1 M dipolar aminon sodium, 0.01 M sodium chloride, 0.001 M EDTA, 0.001 M cysteine, pH 6.0, in Tris buffer, pH 7.6) was purchased from Boehringer Mannheim. Yeast extract and glucose were obtained from Sigma (St. Louis, MO). All other materials were of the highest grade available from Sigma (St. Louis, MO), Fluka, or Merck.

**Yeast strain and culture conditions:** The yeast S. carboxymyces cerevisiae strain hydrogenated by Photorhiza (suspension, 0.5 ml) was cultured aerobically in phosphate – poor medium (15) in glass laboratory fermentor for 15 h at 30°C.

**Yeast purification:** Acid phosphatase was purified from yeast cells as follows. The yeast cells (200 g) were suspended in 0.1 M sodium acetate buffer pH 4.0 and disrupted by shaking at an equal volume of yeast suspension and glass. The cell suspension was passed through a 100 um nylon filter (2 min at 600 rpm) and centrifuged at 2°C. The supernatant was treated with 1% DTT to reduce the activity by 50% and then centrifuged at 2°C. The supernatant was then dialyzed against 0.1 M sodium acetate buffer pH 4.0. After dialysis, the enzyme solution was concentrated by ultrafiltration through Amicon HA-50 membrane. The concentrated enzyme solution was filtered through 0.22 um filter and then chromatographed on DEAE – Sepharose CL-6B (Pharmacia, Uppsala) as described previously for the purification of protease-activated enzyme (16). After the enzyme was eluted from DEAE – Sepharose CL-6B column, the enzyme solution was dialyzed against 0.1 M sodium acetate buffer pH 4.0. The enzyme was then purified by a second chromatography on a mixture of Sephadex G-100 and Sephadex G-1000 (Pharmacia, Uppsala). The enzyme activity was measured as described previously (17).

**Protein determination:** The method of Lowry et al. (17) was employed.

**Yeast assay:** Yeast and human tissues: Yeast samples (1-2 g) were homogenized in the cold in 10 ml of 0.1 M sodium acetate (pH 4.0) and then subjected to centrifugation at 50,000 rpm for 20 min. The supernatant was used for the determination of acid phosphatase activity.

**Electrophoresis:** The method of Weber and Osborn (19) was employed for the electrophoresis of the proteins on polyacrylamide gel. The gels were stained according to the instructions of the producer. Protein standards of known pH were obtained from Sigma (St. Louis, MO), were used for the comparison of the profiles. The gels were stained with Coomassie brilliant blue R-250. The gels were washed with 10% acetic acid and then stained with a 10% solution of Coomassie brilliant blue R-250 in methanol and acetic acid (3:1:1). The gels were then destained with 10% acetic acid.

**Ultracentrifugation:** Ultracentrifugation was performed with a Spinco model E analytical ultracentrifuge and 12 mm (0.7–1 ml) aluminum cells. The solutions were allowed to stand at room temperature and analyzed at various times after the addition of the enzyme. The ultracentrifugation was performed at 2°C. The protein concentrations were determined by the method of Lowry et al. (20). The buffer was used as described by Weber and Osborn (19). The proteins were stained with Coomassie blue according to the instructions of the producer.

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