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, Pierottijeua 6, 41000 Zagreb, Yugoslavia

Acid phosphatase from yeast *Saccharomyces cerevisiae* was purified, and its physicochemical and kinetic properties were investigated. The sedimentation coefficient has been determined to be $s_{20,w} = 13.6 \text{ S}$. The diffusion constant has been found to be $3.9 \times 10^{-7} \text{ cm}^2 \text{s}^{-1}$, and the calculated partial specific volume was $\psi = 0.663 \text{ cm}^3/\text{g}$. 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). 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, still 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**

---

$^*$ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 \( 3.9 \times 10^{-5} \text{ cm}^2 \text{s}^{-1} \) 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 Onckley (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 acid) 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-sulfonfyl 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 (\( A_{278} \)) 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 \( 1.8 \times 10^5 \text{ M}^{-1} \text{cm}^{-1} \).

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 \(-11,000 \text{ degrees cm}^2 \text{ dmol}^{-1} \). 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
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).

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. 5. Cross-linking of acid phosphatase with glutaraldehyde. 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% glutaraldehyde; 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.

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-
Properties of Yeast Acid Phosphatase

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>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
<tr>
<td>Ammonia</td>
</tr>
<tr>
<td>Glucosamine</td>
</tr>
</tbody>
</table>

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 (30)) 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 nonequilibrating 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 yeasts 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).

3 S. Barbarić, 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 NH₂-terminal amino acid was detected; but this could be explained by an identical NH₂-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 $K_m$ 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 $\alpha$-helical structure in acid phosphatase in comparison with other investigated glycoproteins which are usually either devoid of $\alpha$-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 sulfhydryl 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.

Acknowledgments—We are indebted to Prof. I. O. Lampen (NJ) and Dr. C. Gancedo (Madrid) for critical reading of the manuscript and to Prof. W. Tannen (Regensburg) for helpful discussions. We wish to thank Prof. P. L. Luisi (Zürich) for the use of a CD spectrometer and fluorometer and for discussion of the spectroscopic work.

REFERENCES
22. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70-75
Properties of Yeast Acid Phosphatase


SUPPLEMENTAL MATERIAL

Title: Physico-chemical and kinetic properties of acid phosphatases

Authors: Slobodan Barbasid Break0

EXPERIMENTAL PROCEDURES

Chemicals - All chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma (St. Louis, MO), and/or Boehringer (Mannheim, Germany). Yeast extracts were purchased from Sigma (St. Louis, MO). Enzyme standards were obtained from Sigma (St. Louis, MO). All other chemicals were of the highest grade available from Sigma (St. Louis, MO).

Yeast Strain and Culture Conditions - The yeast Saccharomyces cerevisiae strain (S. cerevisiae, cerevisiae) was cultured aerobically in phosphate buffer containing 48 ml of yeast extract, 48 ml of dextrose (50%), and about 48 ml of ammonium acetate (50%).

Chromatographic Procedures - Sephadex G-200 was washed with 0.1 M sodium phosphate buffer pH 7.0 and dialyzed against 0.1 M sodium acetate buffer pH 7.0 for 24 h at 30°C. After dialysis, the enzyme solution was concentrated by ultrafiltration through Amicon XM-50 membrane. The concentrated enzyme solution was filtered on a filter aid (Amicon XM-50 membrane).

Analytical Measurements - The ultraviolet spectrum of the enzyme was obtained against 0.1 M sodium acetate buffer pH 7.0. The specific extinction at 280 nm was measured.

Fig. 1 Electrophoresis analysis of acid phosphatase at pH 6.4. 50 µg of acid phosphatase was applied on gel and the gels were stained with Coomassie Brilliant Blue R-250. One gel was obtained using an Analytical gel spectrometer. Fluorescence measurements were performed on a spectrofluorimeter (Jenway 3500/4). The co-electrophoresis procedure is described in Materials and Methods.

Fig. 2 Effect of substrate concentrations on initial velocity of acid phosphatase.
Physicochemical and kinetic properties of acid phosphatase from Saccharomyces cerevisiae.
S Barbaric, B Kozulic, B Ries and P Mildner


Access the most updated version of this article at http://www.jbc.org/content/259/2/878

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/2/878.full.html#ref-list-1