Purification and Characterization of a Secreted Purple Phosphatase from Soybean Suspension Cultures

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ABSTRACT

We purified and partially sequenced a purple (λmax = 556 nanometers) acid phosphatase (APase; EC 3.1.3.2) secreted by soybean (Glycine max) suspension-culture cells. The enzyme is a metalloprotein with a Mn2+ cofactor. This APase appears to be a glycoprotein with a monomer subunit molecular weight of 58,000 and an active dimer molecular weight of approximately 130,000. The protein has an isoelectric point of about 5.0 and a broad pH optimum centered near 5.5. The purified enzyme, assayed with p-nitrophenyl phosphate as the substrate, has a specific activity of 512 units per milligram protein and a Km of approximately 0.3 millimolar; phosphate is a competitive inhibitor with a Km of 0.7 millimolar. This APase is similar to one found in soybean seed meal but dissimilar to that found in soybean seedlings.

This enzyme is similar to an APase found in germinating soybean seeds (8) but different from an APase found in older seedlings (28).

MATERIALS AND METHODS

Cell Line and Growth Conditions

Soybean suspension-culture cells (root-derived, nonphoto-synthetic, line SB1, Glycine max L. Merr.) were grown in 1B5 media (9), which contains 1.1 mM NaH2PO4 and 1 ppm 2,4-D. Cultures were subcultured twice weekly.

APase Enzyme Assays

Unless otherwise indicated, APase assays were performed in 5 mM sodium acetate buffer (pH 5.5) containing 5.0 mM p-nitrophenyl phosphate. One milliliter reactions were stopped by the addition of 1.6 mL 1 M NaCO3. The amount of p-nitrophenol (and therefore phosphate) produced was determined by measuring the absorbance of the stopped reaction at 405 nm. One unit of activity is defined as the amount of enzyme causing production of 1 μmol of p-nitrophenol/min at 22°C. Assays were typically done for 5 min. Assays were linear with time up to 8 min (r > 0.93) and with enzyme concentration between 0.08 and 8 μg/mL (r > 0.9).

Purification of APase

Up to 1 kg of cells per batch was collected by filtration through Whatman No. 2 filter paper at room temperature. All remaining operations were done at 4°C unless otherwise specified. Cells were treated with extraction buffer (0.5 M CaCl2 in 5 mM Hepes-Mes, pH 7.8; 1 mL buffer/g fresh weight) at 4°C for at least 1 d. The extraction buffer was removed by filtration and mixed with an equal volume of cold acetone to precipitate proteins. Different batches were combined at this stage. The precipitate was recovered (5000 g, for 1 h at 10°C) and stored at −90°C in 60% (v/v) glycerol, 5 mM Hepes-Mes (pH 7.0) or rediluted for ammonium sulfate precipitation. The precipitate from a 35 to 75% saturation ammonium sulfate solution was collected, brought up in buffer A (35 mM acetate buffer, pH 4.0), and dialyzed against the same buffer containing 0.02% (w/v) sodium azide. The sample was loaded onto a carboxymethyl cellulose column (2.5 × 11.5 cm) equilibrated in buffer A. Proteins were eluted from the column using a flow rate of 2.25 mL/min with a linear 160 mL 10 mM to 1 M NaCl gradient in buffer A. Peak fractions were adjusted to pH 6.0 using sodium acetate and

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2 Abbreviation: APase, acid phosphatase.
brought to 100 mM NaCl, 1 mM MnCl₂, 1 mM CaCl₂, and 1 mM MgCl₂, pH 6.0, and dialyzed against Con A buffer (100 mM sodium acetate, 500 mM NaCl, 1 mM MnCl₂, 1 mM CaCl₂, and 1 mM MgCl₂, pH 6.0). A column (0.75 × 10 cm) of Con A Sepharose (Sigma) was equilibrated in Con A buffer, and approximately 2 mg of APase preparation was passed over the column twice. The column was then washed with 6 mL of Con A buffer to remove any loosely associated proteins. Bound proteins were eluted at a rate of 1 mL/min with Con A buffer containing 1 M α-methylmannopyranoside (Sigma) and 0.5 mM glucose (50 mL total elution volume). Peak APase fractions eluted from the carboxymethyl cellulose column were also dialyzed against buffer A containing 1 M NaCl and 2 mL sample volume applied to a 1.5 cm × 1 M BioGel A1.5M column (Bio-Rad). Proteins were eluted with buffer A containing 1 M NaCl at a flow rate of 0.5 mL/min. Proteins were assayed using the techniques of Bradford (3) and Lowry et al. (16) using BSA as a standard. Purity was checked using SDS-PAGE (14); molecular mass was calculated using log plots of molecular radius and migration of mol wt standards (Sigma). Activity stains of gels were performed as described (30).

Amino Acid Composition and Sequence

Purified APase was sequenced by the Texas A&M Biotechnology Support Laboratory on an Applied Biosystems automatic gas-phase sequencer, model 470A. Amino acid composition from approximately 7 nmol of purified APase was determined with a Waters Picotag system.

Atomic Absorption Spectroscopy

Atomic absorption spectroscopy was performed on an Instrumentation Laboratory S-12 flame spectrophotometer by Soil Analytical Services, Inc. (College Station, TX).

Isoelectric Focusing

A 12 mL protein sample (approximately 5 mg) dialyzed against water was added to enough chromatofocusing buffer (10% glycerol, 4 mM urea, 2% Bio-Rad broad pH range ampholytes) to give a total volume of 55 mL. This sample was run on a Bio-Rad Rotofor preparative scale chromatofocusing column and run at 12 W constant power until the voltage peaked (1500 V). Twenty fractions of approximately 2.5 mL each were collected and assayed for APase activity and pH.

RESULTS

Purification of APase

Itonically-bound cell wall APase was extracted from the surface of soybean suspension-culture cells by washing cells with 0.5 mM CaCl₂ and purified to homogeneity (Table I). Salts of monovalent cations were also tested, but as reported by Nagahashi and Seibels (20), calcium salts were more effective in extracting wall-bound APase. Activity gels stained for APase activity show that only a single form of APase is present in soybean suspension-culture cell walls (not shown; an identical activity band is found in total cell homogenates and in spent media, R. Record and L.R. Griffing, unpublished results). The majority (98%) of APase activity remains associated with the cells after CaCl₂ extraction, but this step provides a more than 100-fold purification (Table I). APase was precipitated from a 35 to 75% ammonium sulfate solution, dialyzed, and centrifuged to remove contaminating polysaccharides. Ion-exchange chromatography over carboxymethyl cellulose gave further purification, at times providing up to threefold purification depending on the initial wall extract. Final purification was achieved on a Con A column (Table I). Chromatography on phosphocellulose did not further purify the APase. Molecular sieving on an A1.5M column identified the native form of the APase as a protein of approximately 130 kD (data not shown).

Composition of the Active Form of APase

APase activity was retained on a Con A-Sepharose column, and most of this activity eluted with 1 M α-methylmannnoside. This indicates that APase may be a glycoprotein.

SDS-PAGE of purified protein showed that the APase is homogeneous with a subunit molecular mass of 58 kD (Fig. 1, lane A). In activity gels, APase activity appears as a broad

Table I. Purification of Soybean-Secreted APase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Units</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Purification</th>
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<tr>
<td>Cells</td>
<td>1,120,000</td>
<td>1,420,000</td>
<td>0.791</td>
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<tr>
<td>CaCl₂ wash</td>
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<td>197</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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<td>54.6</td>
<td>147</td>
<td>186</td>
</tr>
<tr>
<td>CMC*</td>
<td>457</td>
<td>2.8</td>
<td>326</td>
<td>412</td>
</tr>
<tr>
<td>Con A</td>
<td>179</td>
<td>0.35</td>
<td>512</td>
<td>647</td>
</tr>
</tbody>
</table>

* Carboxymethyl cellulose.

Figure 1. SDS-PAGE (12.5% [w/v] polyacrylamide) analysis of purified APase. Lane A, Purified APase (1.3 μg, purified as outlined in Table I) stained with silver (Bio-Rad). Only a single protein band at 58 kD is visible. Lane B, Darts show high molecular mass standards at 205, 116, 97, 66, 45, and 29 kD (Sigma).
The violet color of APase is apparent from its visible absorption spectrum, which shows that the protein absorbs maximally in the middle of the visible region (peaking at 556 nm). The lower curve shows that dithionite, which bleaches iron-containing APases, has no effect. Similar results were reported for an APase isolated from soybean seeds (8).

Atomic absorption spectroscopy showed the presence of approximately 1 atom of manganese per 58-kD subunit of protein. No iron or zinc was associated with the protein. In contrast with the purple acid phosphatase of red kidney bean, the 556-nm absorbance peak persists in the presence of dithionite (1) (Fig. 2), confirming the absence of iron in the chromophore of the enzyme.

Figure 2. Visible absorbance spectrum. The physical basis for the violet color of APase is apparent from its visible absorption spectrum, which shows that the protein absorbs maximally in the middle of the visible region (peaking at 556 nm). The lower curve shows that dithionite, which bleaches iron-containing APases, has no effect. Similar results were reported for an APase isolated from soybean seeds (8).

Analysis of APase Activity

APase has a broad pH optimum with a maximum at approximately pH 5.5 (Fig. 3). The average K_m determined from four determinations was 0.3 mM (Fig. 4). The slope of less than 1 (0.65) on a Hill plot (Fig. 5) indicates negative cooperativity with regard to substrate binding.

Phosphate inhibited the APase activity, as shown in Figure 4. A Dixon plot of the kinetics of APase hydrolysis of p-nitrophenyl phosphate shows inhibition by inorganic phosphate (Fig. 6), with a K_i of 0.7 mM. The nature of the inhibition was competitive (Fig. 4).

Amino Acid Composition and Sequence

Amino acid composition was determined (Table II), and as is the case for APases from tomato (23), sunflower (22), and
wheat (29), the soybean APase is high in glycine, serine, and acidic residues, and low in methionine and basic residues. Subunit molecular mass calculated from the amino acid composition is consistent with a value of 58 kD.

Partial amino acid sequence from the N terminus determined the first 17 amino acids to be K-V-E-K-A-V-D-M-P-L-D-S-D-V-F-A-R. The generation of a single sequence implies either that one of the 58-kD subunits is blocked or that they are nearly identical in sequence.

**DISCUSSION**

Secretry APases also have been purified from sunflower (22) and sycamore (4). These APases initially have molecular mass of around 100,000 D although upon purification, the sunflower APase is cleaved into fragments of 56 and 52 kD (22), and the sycamore APase is cleaved into fragments of 59 and 35 kD (4). Our secreted APase has a similar mol wt (active native form of 130 kD), and is apparently a homodimer of 58-kD monomers. Goldstein et al. (11) reported that the phosphate starvation-induced APase secreted by tomato suspension cells is 57 kD and has a tendency to form higher molecular mass aggregates. A 55-kD Mn⁺⁺-containing APase has been purified from sweet potato (26). It is possible that the initial high molecular mass of sycamore and sunflower APases reflects the common propensity for glycoproteins to aggregate, or that the higher mol wt forms represent dimers similar to those of the soybean APase. We have not yet ruled out the possibility that the 58-kD band represents identically sized degradation products of the 130-kD APase, and that one of these is blocked at the N terminus.

Silver staining of the purified protein shows a single band on SDS-PAGE (Fig. 1). During purification, the ammonium sulfate step helps to eliminate polysaccharide contaminants but does not significantly increase specific activity (Table I). The carboxymethyl cellulose step provides a two- to threefold purification. The final specific activity (512 units/mg protein; Table I), although lower than that found in mustard suspension culture (1225 units/mg protein, ref. 6), is similar to a value reported recently for a wheat germ acid phosphatase (605 units/mg protein, ref. 29).

The purple phosphatase secreted by soybean suspension-culture cells is similar or identical to a purple phosphatase purified from imbimed soybean seeds (8), but distinct from an acid phosphatase (not purple, not glycosylated, lower pH optimum, lower $K_m$) purified from germinating seedlings (28). The relationship between all of the previously reported APases, and a more rigorous assessment of phosphate and developmental regulation of APase gene expression, will be possible with the antibody and nucleic acid probes that are being produced.

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**LITERATURE CITED**