Isolation and Some Properties of Acid Phosphatase-1 from Tomato Leaves

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An isozyme of acid phosphatase-1, acid phosphatase-11, was purified from the leaves of tomato (Lycopersicon esculentum) to homogeneity and characterized. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate. The gel filtration analysis showed that the native molecule had a relative molecular mass of about 61 kilodaltons (kDa). The relative molecular mass of the subunit on gel electrophoresis with sodium dodecyl sulfate was about 32 kDa, indicating that the native form of the enzyme was a homodimer. It was suggested by periodic acid-Schiff staining on the gel that the enzyme was a glycoprotein. The Km for p-nitrophenylphosphate was 2.9 x 10^-3 m. The enzyme had a pH optimum of 4.5 in 0.15 M potassium acetate buffer with p-nitrophenylphosphate as a substrate. This enzyme was activated by divalent metal ions, such as Zn2+, Mg2+, and Mn2+. The N-terminal amino acids were sequenced after the purified enzyme was treated with pyroglutamylpeptidase. It was suggested that the N-terminal amino acid was pyroglutamate.

Acid phosphatases (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.2) are widely distributed in the plant kingdom.1–7) These enzymes are considered to act during germination for the mobilization of phosphorus reservation.8,9) However, only a few enzymes have been purified to homogeneous states.10–14) Since higher plants are known to contain several kinds of Apases of different properties, it is desirable to obtain a homogeneous enzyme preparation for analysis of each enzyme.

In the tomato (Lycopersicon esculentum Mill.), there are several Apases, and Apase-1, one of them, has two electrophoretically separable isozymes15) encoded by two alleles. Apase-11 encoded by the Aps-1 allele is thought to be derived from the wild-type species, Lycopersicon peruvianum and the Aps-11 allele is closely linked to Mi, which confers resistance to the root-knot nematode, Meloidogyne incognita. The other allele, Aps-1*, is thought to be indigenous to L. esculentum, and is not linked to Mi. Therefore, the detection of the Apase-11 isozyme in tomato serves as a selection marker for identifying nematode resistance.16)

For this study, we purified and characterized Apase-11 from leaves of a nematode-resistant strain of L. esculentum Mill. cv VFNT having the Aps-1 and Mi alleles derived from L. peruvianum (Aps-11, Mi).

Materials and Methods

Materials and chemicals. The leaves of tomatoes were obtained from L. esculentum Mill. cv VFNT (Aps-1*, Mi) or L. esculentum Mill. cv Kagome 70 (Aps-1*). The seeds of tomatoes were planted in a greenhouse and grown for 40 to 50 days. The true leaves were cut and used for purification of Apase-1.

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Abbreviations: Apase, acid phosphatase; FPLC, fast performance liquid chromatography; HPLC, high performance liquid chromatography; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; PCMB, p-chloromercuric benzoate; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate.
β-Naphthylphosphate, Fast Black K salt, and Tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co., St. Louis, MO. CM-sephadex C-50, DEAE-Seephadex A-50, ConA-Sepharose, and a Mono P HR 5/20 prepacked fast performance liquid chromatography (FPLC) column were purchased from Pharmacia Fine Chemical, Uppsala. Hydroxyapatite was obtained from Wako Pure Chemical Industries, Ltd., Osaka. Ultrafiltration membranes were purchased from Amicon Co., Panvers, MA. A gel filtration column, TSK gel G3000 SW, was purchased from Tosoh Co., Ltd., Tokyo. Protein assay reagents were obtained from Pierce Co., Rockford, II. All the other chemicals were of the purest grade commercially available.

**Enzyme and protein assay.** Apase activities were assayed at 31°C, pH 5.0, by measuring the rate of liberation of p-nitrophenol from p-nitrophenylphosphate. An assay mixture contained 150 mmol of potassium acetate, 30 mmol of p-nitrophenylphosphate, and enzyme preparation in a total volume of 1.0 ml. After incubation for 30 min, the solution was mixed with 1 ml of 2 N Na₂CO₃ and the absorption of this solution was measured at 410 nm against a blank prepared as described above but lacking enzyme preparation. One unit of the enzyme activity was expressed in μmol of substrate hydrolyzed per min. Specific enzyme activity was expressed as units of enzyme activity per mg of protein.

Protein was measured colorimetrically by the bicinchoninic acid–protein assay system (Pierce Co.) with bovine serum albumin as standard. All spectrophotometric measurements were done with a Hitachi Model 200-10 spectrophotometer. Effluent fractions from column chromatographic procedures were monitored spectrophotometrically for protein content by measuring the absorbance of the solution at 280 nm.

**Gel electrophoresis.** SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) using 4% stacking and 10% resolving gel of 1 mm thickness was done as described by Laemmli[17] and proteins were stained with Coomassie brilliant blue R-250. The relative molecular mass of the subunit of the purified enzyme in the presence of SDS was estimated by comparing its relative mobility with those of standard proteins of known relative molecular mass, α₂-macroglobulin (170 kDa), phosphorylase b (94.7 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa) and trypsin inhibitor (20.1 kDa).

Polyacrylamide slab gel electrophoresis (PAGE) was done as SDS-PAGE without SDS an Apase was stained for activity.18) Electrophoresis was run for 1.5 hr at 20 mA with 24 mm Tris–HCl buffer, pH 8.8, plus 123 mM glycine using an electrophoresis apparatus (Tefco Corporation, Tokyo). Staining was done at 37°C for 30 min by placing a gel in 100 ml of a solution containing 1 mg/ml Fast Black K, 1 mM MgCl₂, 10 ml of 1 M potassium acetate buffer, pH 5.0, and 2.5 ml of 0.1 mM β-naphthylphosphate (dissolved in acetone: water = 1:1).

To detect the Apase-1 activity in tomato, 1 g of leaves was homogenized in a pestle and mortar with 1 ml of 50 mM Tris–HCl buffer, pH 6.8. After centrifugation at 15,000 × g for 5 min in a microtube, 15 μl of the supernatant was analyzed by PAGE. In each step of the purification procedure, a small portion of each fraction was directly analyzed by SDS-PAGE and/or PAGE.

**Gel filtration of TSK gel G3000 SW HPLC.** The purified Apase-1 was filtered on a G3000 SW HPLC gel column using 50 mM Tris-HCl buffer, pH 7.5, plus 0.3 M NaCl at a flow rate of 0.45 ml/min. Apase-1 elution volume was confirmed by activity assay. The proteins used to calibrate the relative molecular mass were: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.3 kDa), all obtained from BioRad.

**Sequencing of the N-terminal amino acids.** Pyroglutamylpeptidase (0.05 units) was added to 500 ng of purified Apase-1 in 35 μl of 50 mM Tris–HCl, pH 8.0, plus 10 mM 2-mercaptoethanol. This mixture was reacted under N₂ gas for 6 hr at 37°C and the solvent was changed to 50 mM trimethylamineacetate buffer, pH 8.0, with Centri-con 10 (Amicon) to remove 2-mercaptoethanol. Then, Apase-1 was degraded by the automated Edman method with an amino acid sequencer (Applied Biosystems 337) as described by Hewich et al.19) Each PTH-amino acid was measured by an HPLC attached to the sequencer.

**Purification of Apase-1.** All manipulations were done at 0-4°C.

**Step 1.** Fresh tomato leaves (3.0 kg) were ground with a pestle and mortar, and homogenized with 6 l of 50 mM Tris–HCl buffer, pH 6.8, in the presence of 0.3 kg of sea sand. The homogenate was filtered under a doubled gauze and centrifuged at 9,000 × g for 30 min. The supernatant, designated crude extract, contained 44.5 g of total protein and 4300 units of Apase activity.

**Step 2.** Solid ammonium sulfate was added to the crude extract to make 40% saturation, stirred, and then placed for 2 hr at 0°C. This suspension was centrifuged at 9,000 × g for 30 min. The supernatant was brought to 80% ammonium sulfate saturation. This suspension was kept at -20°C till use for the next step. Five liters of this suspension were centrifuged at 9,000 × g for 30 min. The supernatant was brought to 80% ammonium sulfate saturation. This suspension was kept at -20°C till use for the next step. Five liters of this suspension were centrifuged at 9,000 × g for 30 min. The pellet was dissolved in 600 ml of 50 mM Tris–HCl buffer, pH 6.8, and the solution was dialyzed against the same buffer for two days. It was then centrifuged at 10,000 × g for 20 min and the precipitate was discarded.

**Step 3.** The supernatant was put on a DEAE-Sephadex A-50 column (4.5 × 45 cm) which had been equilibrated with 50 mM Tris–HCl buffer, pH 6.8. After being washed with the same buffer, the column was eluted with a linear gradient of 0 to 0.66 M NaCl in 50 mM Tris–HCl buffer, pH 6.8. The activity of Apase-1 analyzed by PAGE appeared
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at the vicinity of the 0.4 M-NaCl fractions. The fractions with enzyme activity were pooled and concentrated using an ultrafiltration membrane (Amicon YM-10). This solution was dialyzed against 50 mM potassium acetate buffer, pH 5.0, overnight.

Step 4. A CM-Sephadex C-50 column (4.5 x 45 cm) was equilibrated with 50 mM potassium acetate buffer, pH 5.0. The enzyme solution was put onto this column, and after being washed with the same buffer, the enzyme bound to the column was eluted with a linear gradient of 0 to 0.4 M NaCl in 50 mM potassium acetate buffer, pH 5.0. The activity of Apase-1 appeared around the 0.15 M-NaCl fractions. The fractions with this enzyme activity were pooled and concentrated using an ultrafiltration membrane (Amicon YM-10). The concentrated sample was dialyzed against 10 mM potassium phosphate buffer, pH 7.0.

Step 5. A hydroxyapatite column (2.6 x 10 cm) was equilibrated with 10 mM potassium phosphate buffer, pH 7.5. The enzyme sample was put on this column and after being washed with the same buffer, the enzyme was eluted with a linear gradient of 0 to 500 mM potassium phosphate buffer, pH 7.0. The fractions with the enzyme activity were pooled and concentrated using an ultrafiltration membrane (Amicon YM-10). The concentrated sample was dialyzed against 10 mM potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl, 1 mM MgCl₂, and 1 mM CaCl₂ overnight.

Step 6. A ConA-Sepharose column (1.6 x 8 cm) was equilibrated with 10 mM potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl, 1 mM MnCl₂, and 1 mM CaCl₂. The sample was put onto this column, then after the run-through fraction was eluted, the buffer was changed to 50 mM Tris-HCl buffer, pH 7.0, containing 0.2 M NaCl. In this step, most of the Apase-1 was eluted with a small amount of another Apase, probably Apase-2. After Apase-1 was eluted, most of the other Apases were eluted with a linear gradient of 0 to 0.2 M glucose in 50 mM Tris-HCl buffer, pH 7.0, containing 0.2 M NaCl. The fractions with Apase-1 activity were pooled and concentrated using an ultrafiltration membrane (Amicon YM-10).

Step 7. A ConA-Sepharose column was equilibrated with 50 mM Tris-HCl buffer, pH 7.0, containing 0.2 M NaCl. The concentrated sample was applied to this column again. After being washed with the same buffer, the enzyme bound to the column was eluted with 50 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. Almost homogenous Apase-1 was eluted after the buffer change. The fractions with the enzyme activity were pooled, concentrated, and dialyzed against 25 mM bisTris-iminodiacetate buffer, pH 7.1, as described above. These two steps of ConA-Sepharose chromatography were very effective to separate Apase-1 from the other Apases in tomato leaves, since Apase-1 had a lower affinity for the ConA-Sepharose column than the other Apases.

Step 8. The sample was put onto a Mono P column (HR5/20) which had been equilibrated with 25 mM bisTris-iminodiacetate buffer, pH 7.1. After being washed with the sample buffer, the enzyme bound to the column was eluted with 10-fold diluted polybuffer (Pharmacia) adjusted to pH 4.0 with iminodiacetic acid.

**Results and Discussion**

**Purity of the enzyme and its molecular weight**

In a preliminary experiment, we confirmed the data reported by Rick and Fobes that Apase-1 can be distinguished from the several other Apases which are present in tomatoes by using electrophoresis as shown in Fig. 1. Then Apase-1 was purified from tomato leaves. The efficiency of purification could not be measured by calculating the specific activity, because it was impossible to measure only Apase-1 activity independently from those of the other Apases. The peak of the enzyme activity coincided with the peak of the protein in Step 8, suggesting that the enzyme was purified and that its pi was 4.2 (Fig. 2). As a result, Apase-1 (0.687 mg protein and 14.7 units) was obtained from 10 kg of tomato leaves and the specific activity was 21.4 units/mg protein. The final preparation of the enzyme was electrophoresed by SDS-PAGE and PAGE. The purified enzyme yielded a single band in each analysis (Figs. 3A and 3B), the relative molecular mass of the subunit was 32 kDa from the position of the

![Fig. 1. The Isozymes of Apase-1 of Tomato.](image)

A crude extract from tomato leaves was analysed by polyacrylamide gel electrophoresis. Lane 1, *L. esculentum* Mill. cv VENT; lane 2, *L. esculentum* Mill. cv Kagome 70. The positions of Apase-1 and Apase-1⁺ are indicated by the arrows.
Fig. 2. Protein Elution Profile in Chromatofocusing on Mono P (FPLC).
Fractions (1.5 ml each) were collected at a flow rate of 0.5 ml/min. Protein concentration monitored at A$_{280}$nm (--); pH gradient, (-----); Apase activity (●—●).

Fig. 3. SDS-PAGE and PAGE of purified Apase-1.  
Purified Apase-1 was chromatographed by SDS-PAGE (A, C) and PAGE (B). (A): Protein was stained with Coomassie brilliant blue. Lane 1, molecular weight markers; lane 2, purified Apase-1. (B): The enzyme activity was stained. Lane 1: purified Apase-1. (C): The enzyme was stained with the periodic acid-Schiff’s reagent. Lane 1, molecular weight markers; lane 2, purified Apase-1.

band in Fig. 3A, and the enzyme on SDS-PAGE gel gave a colored band when it was stained with periodic acid-Schiff’s reagent (Fig. 2C). This latter result together with the behavior of the enzyme in a ConA-Sepharose column suggested that this enzyme was a glycoprotein. The relative molecular mass of native Apase-1 was found by HPLC gel filtration to be about 61 kDa relative to the standard markers (Fig. 4), and it was suggested that the native form of Apase-1 was a homo-dimer. During the course of this investigation, Paul et al. reported the purification of Apase-1 from cultured tomato cells. They showed that the relative molecular mass of the native form is about 51 kDa, which is smaller than our result. The reason for this discrepancy is not clear, but the lengths of glycoside chains at the glycosylation sites of the enzyme might be different between the enzymes from cultured cells and from tomato leaves.

**Optimum pH and Km**

The optimum pH for the activity with p-nitrophenylphosphate as a substrate in po-
The *Km* for *p*-nitrophenylphosphate in potassium acetate buffer, pH 5.0, was $2.9 \times 10^{-3}$ M (Fig. 6).

**Fig. 4.** The Relative Molecular Mass of Native Apase-1

The relative molecular mass of native Apase-1 was measured by an HPLC gel filtration column G-3000SW. A, thyroglobulin (670 kDa); B, γ-globulin (158 kDa); C, ovalbumin (44 kDa); D, myoglobin (17 kDa); E, VB-12 (1.3 kDa). The position of Apase-1 is indicated by the arrow.

**Fig. 5.** The Effects of pH on the Activity of Apase-1.

A410 represents Apase activity with *p*-nitrophenylphosphate as substrate assayed at different pHs for 30 min at 31°C.

**Km** = $2.9 \times 10^{-3}$

substrate : *p*-nitrophenylphosphate

**Fig. 6.** *Km* for Apase-1 with *p*-Nitrophenylphosphate as Substrate.

Lineweaver-Burk plots against *p*-nitrophenylphosphate concentration in the range of 5–30 mM are shown. A410 represents Apase activity with different concentration of *p*-nitrophenylphosphate as substrate assayed at pH 5.0 for 30 min at 31°C.

**Effectors**

The data in Table I show that the activity of the enzyme with *p*-nitrophenylphosphate as a substrate was not significantly influenced by PCMB, NaF, CaCl2, or Na2MoO4. Furthermore, the result shown in Table I indicates that this enzyme activity was not
Table I. Effects of Several Compounds on the Activity of Purified Acid Phosphatase-1 from Tomato Leaves

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mm)</th>
<th>Relative activity (%)</th>
</tr>
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<tbody>
<tr>
<td>PCMB</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>NaF</td>
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<td>CaCl₂</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>1</td>
<td>106</td>
</tr>
</tbody>
</table>

affected by a reaction product, NaH₂PO₄. This is a clear contrast with the results described for some other Apases isolated from plants.⁵¹⁰¹³* EDTA and HgCl₂ inhibited the activity by 33% and 36%, respectively, while MgSO₄, ZnSO₄, and MnCl₂ activated the activity several-fold. It was suggested that the activities of Apases from rice and from sweet potato were enhanced by divalent ions and that Apase from spinach²²) was activated by Cu²⁺ ions after once being inactivated by dialysis. It is speculated that Apase-1 from tomato leaves may have Zn²⁺ ions as a cofactor or a ligand, since Zn²⁺ ion gave the highest stimulative effect on the enzyme activity and the enzyme was colorless.

The N-terminal amino acid sequence

The N-terminal amino acid residue of the purified native enzyme could not be released by the Edman degradation. So it was treated with pyroglutamylpeptidase, then the sequence was found to be D-E-L-K-x-T-T-W-R-F-V-V-E-T (x is an unidentified amino acid). It is speculated from our result that the N-terminal amino acid of Apase-1 from tomato leaves may be pyroglutamic acid and that the x in this sequence may be an aspargine that is blocked by glycosylation. This information on the N-terminal amino acid sequence of the enzyme is sufficient to synthesize oligonucleotides corresponding to this region of the enzyme and to clone cDNA encoding the enzyme, which will be useful to clone a DNA fragment containing the Mi locus and to analyze the linkage between the Ap-1 and Mi alleles in L. esculentum Mill. cv. VFNT.

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References