

Purification and Characterization of a Potato Tuber Acid Phosphatase Having Significant Phosphotyrosine Phosphatase Activity¹

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The major acid phosphatase (APase) from potato (*Solanum tuberosum* L. cv Chiefton) tubers has been purified 2289-fold to near homogeneity and a final O-phospho-L-tyrosine (P-Tyr) hydrolyzing specific activity of 1917 $\mu\text{mol Pi produced min}^{-1} \text{mg}^{-1}$ of protein. Nondenaturing polyacrylamide gel electrophoresis of the final preparation resolved a single protein-staining band that comigrated with APase activity. Following sodium dodecyl sulfate polyacrylamide gel electrophoresis, glycosylated polypeptides of 57 and 55 kD were observed. The two polypeptides are immunologically closely related, since both proteins cross-reacted on immunoblots probed with rabbit anti-(*Brassica nigra* APase) immunoglobulin G. Immunoblotting studies revealed that the 55-kD subunit did not arise via proteolytic cleavage of the 57-kD subunit after tissue extraction. The native molecular mass was approximately 100 kD, suggesting that the holoenzyme could exist as either a homodimer or a heterodimer. The enzyme displayed a pH optimum of 5.8, was activated 40% by 4 mM Mg^{2+} , and was potently inhibited by molybdate, vanadate, and ZnCl_2 . The final preparation displayed the highest activity and specificity constant with P-Tyr, but also dephosphorylated other phosphomonoesters including p-nitrophenylphosphate, O-phospho-L-serine, phosphoenolpyruvate, PPI, and ATP. Antibodies to P-Tyr were used to demonstrate that several endogenous phosphotyrosylated tuber polypeptides could serve as in vitro substrates for the purified APase. Although the precise physiological significance of the potato APase's substantial in vitro activity with P-Tyr remains obscure, the possibility that this APase may function to dephosphorylate certain protein-located P-Tyr residues in vivo is suggested.

APases (orthophosphoric-monoester phosphohydrolase; EC 3.1.3.2) having broad and overlapping substrate specificities are ubiquitous and abundant enzymes. Intracellular and secreted APases are believed to play a major role in Pi scavenging and the utilization and turnover of Pi-rich sources occurring in either animal lysosomes or plant vacuoles (Hollander, 1971; Duff et al., 1994). Although plant APases exhibit considerable structural and kinetic diversity, they

appear to be immunologically closely related, indicating a common ancestral origin (Duff et al., 1994). Several plant APases, including phytase, 3-phosphoglycerate phosphatase, and PEP phosphatase, display a clear but nonabsolute substrate selectivity (Duff et al., 1994).

Based on work performed with crude preparations, potato (*Solanum tuberosum* L.) tuber APase has been considered as the standard for "nonspecific" APase activity (Alvarez, 1962; Hsu et al., 1966). Subsequent studies indicated that potato APase may exist in up to six isoforms found in the cytoplasmic and cell wall compartments (Kubicz, 1973; Sugawara et al., 1981). Differential degrees of glycosylation may account for much of the observed variability. The major potato APase isoform has been reported to exist as a 96-kD homodimer (Kubicz, 1973). The possibility that potato tuber APase may function as a phosphoprotein phosphatase in vivo is indicated by the routine utilization of the enzyme to dephosphorylate phosphoproteins such as phosphocasein (Bingham et al., 1976) and autophosphorylated protein kinases (Chen and Blenis, 1990). Similarly, the capacity of potato APase to dephosphorylate P-Tyr has been favorably compared to authentic nonplant P-Tyr protein phosphatases (Zhao et al., 1992). Polya and Wettenhall (1992) recently described the purification to homogeneity of a minor 28-kD APase isoform from potato tubers that was inhibited by cyclic nucleotides and efficiently cleaved Pi from P-Tyr (but not P-Ser or P-Thr).

Phosphotyrosyl protein phosphatases (EC 3.1.3.48) have been widely implicated in regulation of the cell cycle and cellular differentiation in nonplant species (Charbonneau and Tonks, 1992; Jagiello et al., 1992). A number of purified mammalian and yeast cytosolic or membrane-bound P-Tyr protein phosphatases have been thoroughly characterized (Lau et al., 1989; Brautigan, 1992; Pot and Dixon, 1992; Barford et al., 1994). Although P-Tyr proteins are present in plant cells at levels (relative to phosphoserine or phosphothreonine) greater than those found in animal cells (Elliot and Geytenbeek, 1985), comparatively little is known about roles or mechanisms of Tyr phosphorylation in plants.

In this report we describe the purification and characterization of the predominant APase from potato tuber. We demonstrate that the enzyme is a stable glycoprotein that

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Abbreviations: APase, acid phosphatase; FPLC, fast-protein liquid chromatography; P-Ser, O-phospho-L-serine; P-Thr, O-phospho-L-threonine; P-Tyr, O-phospho-L-tyrosine.

effectively hydrolyzes Pi from P-Tyr, phosphitin, and potato tuber P-Tyr proteins. The results suggest that the enzyme could function as a P-Tyr protein phosphatase *in vivo*.

MATERIALS AND METHODS

Chemicals and Plant Material

Biochemicals, coupling enzymes, PEG, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, Tween 20, bisacrylamide, and nonprestained SDS-PAGE molecular mass standards were purchased from Sigma. Prestained "rainbow" molecular mass standards were purchased from Amersham (Oakville, Ontario, Canada). Protein assay reagent was obtained from Bio-Rad (Mississauga, Ontario, Canada). Cyanogen bromide was obtained from Kodak (Toronto, Ontario, Canada). Phosphocellulose P-11 was purchased from Whatman (Hillsboro, OR). Polyvinylidene difluoride membranes (Immobilon transfer; 0.45- μm pore size) were supplied by Millipore (Mississauga, Ontario, Canada). S-Sepharose, a Phenyl Superose HR 5/5 column, and an FPLC system were obtained from Pharmacia (Baie D'Urfe, Quebec, Canada). Monospecific, affinity-purified rabbit anti-(P-Tyr) IgG (Kamps and Sefton, 1988) was a kind gift of Dr. Peter Greer (Department of Biochemistry, Queen's University). All buffers used in this study were degassed and adjusted to their respective pH values at 25°C. Mature tubers of potato (*Solanum tuberosum* L. cv Chiefton) were purchased at a local market and used the same day.

Enzyme Assays

Phosphatase Assay A

For routine measurements of APase activity, the hydrolysis of PEP to pyruvate was coupled with the lactate dehydrogenase reaction and assayed at 25°C using a Varian DMS 200 spectrophotometer. Standard assay conditions were 50 mM Na-acetate buffer (pH 5.8) containing 5 mM PEP, 4 mM MgCl_2 , 0.2 mM NADH, and 3 units of dialyzed rabbit muscle lactate dehydrogenase in a final volume of 1 mL. Assays were initiated by the addition of enzyme preparation. One unit of APase activity is defined as the quantity of enzyme that would catalyze the hydrolysis of 1 μmol of substrate/min at 25°C.

Phosphatase Assay B

For substrates other than PEP, the method of Eibl and Lands (1969) was used to detect the Pi released by the APase reaction. APase (0.05 units) was incubated in 1.5-mL cuvettes with 0.9 mL of 50 mM Mes/NaOH (pH 5.8) containing 4 mM MgCl_2 and an alternative substrate (5 mM unless otherwise indicated) for 6 min at 25°C. Reactions were terminated by the sequential addition of 0.1 mL of reagent A (3 M H_2SO_4 containing 20 mM ammonium molybdate) and 10 μL of 1% (v/v) Triton X-100. Samples were incubated at 25°C for 20 min and the A_{660} was determined. To calculate activities, a standard curve over the range of 0.01 to 1.0 μmol of Pi was constructed for each set of assays. Assays were performed in triplicate and controls were run for background amounts of

Pi present at each substrate concentration by adding reagent A before the enzyme. Hydrolysis was proportional to enzyme concentrations between 0.005 to 0.1 unit/mL and remained linear with time for at least 15 min.

Kinetic Studies

Acid-washed cuvettes were used for all kinetic studies. Apparent K_m values were determined from the Michaelis-Menten equation fitted to a nonlinear least-squares regression computer kinetics program (Brooks, 1992). All kinetic parameters are the means of duplicate determinations performed on two separate preparations of the purified enzyme, and they are reproducible to within $\pm 10\%$ SE.

Enzyme Purification and Buffers Used in APase Purification

All purification procedures were carried out at 4°C.

Buffer A consisted of 50 mM Na acetate (pH 4.9) containing 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. Buffer B consisted of 50 mM Na acetate (pH 5.2) containing 1 mM EDTA and 1 mM DTT. Buffer C was 30 mM Hepes/NaOH (pH 6.8) containing 1 mM EDTA. Buffer D was 25 mM bis-tris-propane/HCl (pH 6.5) containing 1 mM EDTA, 0.5 mM DTT, and 30% (saturation) $(\text{NH}_4)_2\text{SO}_4$. Buffer E consisted of 25 mM bis-tris-propane/HCl (pH 6.5) containing 1 mM EDTA, 0.5 mM DTT, and 15% (v/v) ethylene glycol.

Crude Extract

Peeled and diced potato tubers (600 g) were homogenized in 1 volume of buffer A with a Waring blender and a Polytron. The homogenate was squeezed through six layers of cheesecloth, filtered through one layer of Miracloth, and centrifuged at 14,000g for 20 min. Supernatant fractions were pooled and designated the crude extract.

Acidification

The crude extract was adjusted to pH 4.0 with 7 M acetic acid and stirred for 10 min. The extract was adjusted to pH 4.9 with 15 M NH_4OH and centrifuged as above. Pellets were discarded.

Fractionation Using PEG

PEG (average molecular mass 8 kD; 50% [w/v] dissolved in 20 mM bis-tris-propane/HCl [pH 7.4] containing 1 mM EDTA) was added to the supernatant fluid to give a final concentration of 5% (w/v). The solution was stirred for 10 min and centrifuged as above. Pellets were discarded and the supernatant fraction was adjusted to 18% (w/v) PEG-8000 by the slow addition of finely ground powder, then stirred for 40 min and centrifuged as above. The pellets were resuspended in 75 mL of buffer B, adjusted to pH 5.2 with 7 M acetic acid, and clarified by centrifugation at 37,000g for 10 min.

S-Sepharose Chromatography

The clear supernatant fluid was adjusted to a protein concentration of 10 mg/mL with buffer B and absorbed at 1

mL/min onto a column of S-Sepharose (1.5 × 21 cm) that had been pre-equilibrated in buffer B. The column was connected to an FPLC system, washed with buffer B until the A_{280} decreased to less than 0.1, and eluted with a linear 0 to 0.5 M KCl gradient (250 mL) in buffer B (fraction size, 5 mL). Peak activity fractions (eluted at approximately 130 mM KCl) were pooled and concentrated to about 10 mL with an Amicon YM-30 ultrafilter. For desalting, the concentrated S-Sepharose peak fractions were diluted to 45 mL with buffer C, reconcentrated to 10 mL as above, and rediluted to 45 mL with buffer C.

Phosphocellulose Chromatography

Immediately prior to its use, phosphocellulose P-11 was hydrated and precycled according to the manufacturer's instructions. The desalted, pooled fractions from S-Sepharose chromatography were absorbed at 0.5 mL/min onto a column of phosphocellulose P-11 (0.75 × 13.5 cm) that had been pre-equilibrated with buffer C. The column was connected to an FPLC system, washed with buffer C until the A_{280} decreased to less than 0.1, and eluted with a linear 0 to 1 M KCl gradient (250 mL) in buffer C (fraction size 2 mL).

Phenyl Superose FPLC

Solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the pooled peak phosphocellulose fractions (eluted at approximately 450 mM KCl) to bring the final concentration of $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation. The solution was stirred for 30 min and centrifuged at 37,000g for 15 min. The supernatant fluid was absorbed at 0.5 mL/min onto a Phenyl Superose HR 5/5 column pre-equilibrated in buffer D, and the column was washed with this buffer until the A_{280} decreased to baseline. The column was eluted in a stepwise fashion with decreasing concentrations of buffer D and simultaneously with increasing concentrations of buffer E. APase activity was eluted in a sharp peak following a step from 0 to 20% buffer E (100–80% buffer D; fraction size, 1 mL). Pooled peak fractions were concentrated to approximately 600 μL using an Amicon YM-30 ultrafilter and stored at 4°C. APase activity remained constant for at least 6 weeks, when the final preparation was stored at 4°C.

Stability of APase in Potato Tuber Extracts

Clarified extracts were prepared by homogenizing peeled and diced mature potato tuber tissue (0.5 g) with a Polytron in 1 volume of (a) buffer A lacking PMSF; (b) buffer A supplemented with the following protease inhibitors: 1 mM *N* α -*p*-tosyl-L-Lys chloromethyl ketone, 1 mM *N*-tosyl-L-Phe chloromethyl ketone, 1 mM diphenylcarbamylchloride, 2 mM *p*-hydroxymecuribenzoate, 3 mM 1,2-epoxy-3-(*p*-nitrophenoxy)-propane, 10 mM bipyridyl, 5 mM 1,10-phenanthroline, 0.1 mg/mL soybean trypsin inhibitor, 0.1 mg/mL pepstatin, 0.1 mg/mL antipain, and 0.1 mg/mL aprotinin; or (c) hot (90°C) SDS-PAGE sample buffer. The homogenates were centrifuged for 10 min at 16,000g in an Eppendorf microcentrifuge. A 0.5-mL aliquot of the crude supernatant fluid prepared in buffer A lacking protease inhibitors (a) was

incubated for 16 h at 25°C. An aliquot of each crude supernatant fraction was mixed with an equal volume of SDS-PAGE sample buffer and boiled for 3 min for immunoblot analysis using anti-*Brassica nigra* [black mustard] PEP-specific APase IgG as described below.

Electrophoresis and Determination of Native and Subunit Molecular Masses

Nondenaturing PAGE was performed with a Bio-Rad (La Jolla, CA) mini-gel apparatus using the discontinuous system of Davis (1964). The final acrylamide monomer concentration in the 0.75-mm-thick slab gels was 8% (w/v) for the separating gel and 2.5% (w/v) for the stacking gel. Prior to pouring the stacking gel solution, the separating gel was pre-electrophoresed for 2 h at 150 V constant voltage, with 250 mM Tris/HCl (pH 8.8) as the electrode buffer. The stacking gel was polymerized with fluorescent light for 3 to 4 h. Gels were precooled to 4°C and were maintained at this temperature during electrophoresis. Samples containing 50% (v/v) glycerol were run at a constant voltage of 120 V, applied for 2 h. Tris (25 mM)/Gly (190 mM) containing 1 mM thioglycolate was used as the electrode buffer. Gels were either stained for protein using Coomassie blue R-250 or APase activity was located in the gel. To detect APase activity, the gel was equilibrated for 15 min at 24°C in 100 mM Na acetate (pH 5.8) containing 20 mM CaCl_2 , followed by incubation for 15 min at room temperature in 100 mM Na acetate (pH 5.8) containing 20 mM CaCl_2 , 0.02% (w/v) Fast Garnet GBC salt, and 0.02% (w/v) Na_2 -naphthyl phosphate. For second-dimension electrophoresis, the single protein-staining band was excised from the nondenaturing gel and incubated in 62 mM Tris/HCl (pH 6.8) containing 10% (v/v) glycerol and 2% (w/v) SDS for 40 min at 50°C. After equilibration in SDS the gel slice was subjected to SDS-PAGE as described below.

Denaturing SDS-PAGE was performed using a Bio-Rad mini-gel apparatus and the discontinuous system of Doucet and Trifaró (1988) or Laemmli (1970). Final acrylamide monomer concentration in the 0.75-mm-thick slab gels was 14, 12, or 10% (w/v) for the separating gel and 4% (w/v) for the stacking gel. All samples were preincubated in the presence of SDS sample buffer (70 mM Tris/HCl, pH 6.7, containing 8 M urea, 3% [w/v] SDS, 100 mM DTT, and 0.005% [w/v] bromophenol blue) for 3 min at 100°C prior to being loaded on the gels. Gels were run at a constant voltage of 155 V, applied for 1 h. For the determination of subunit molecular masses by SDS-PAGE, a plot of relative mobility versus log (molecular mass) was constructed with the following standard proteins: β -galactosidase (116 kD), phosphorylase *b* (97.4 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). Glycoprotein staining of SDS gels was conducted using a periodic acid-Schiff silver-staining protocol (Dubray and Bezard, 1982).

Nondenaturing SDS-PAGE was utilized for native molecular mass determinations (Goldstein et al., 1988). SDS-PAGE was carried out as described above except that the purified APase was mixed with 1% (w/v) SDS and 0.005% (w/v) bromophenol blue without boiling. For the determination of native molecular mass by SDS-PAGE, a plot of relative mobility versus log (molecular mass) was constructed with

the following prestained "rainbow" standard proteins: myosin (200 kD), phosphorylase *b* (97 kD), BSA (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), and soybean trypsin inhibitor (21.5 kD).

Peptide Mapping by Cyanogen Bromide Cleavage

Polypeptides were excised individually from an SDS mini-gel and cleaved in situ with cyanogen bromide, and the degradation products were analyzed on a 1-mm-thick 14% (w/v) SDS mini-gel according to the method of Plaxton and Moorhead (1989).

Immunoblotting

Electroblotting was performed as previously described (Moorhead and Plaxton, 1990), with the addition of 100 μ M *o*-vanadate to the transfer buffer when maintenance of phosphotyrosyl residues was desired. All immunoblots to be probed with the anti-(black mustard PEP-specific APase) IgG (Duff et al., 1991) were first pretreated with sodium *m*-periodate according to Laine (1988) so as to oxidize antigenic oligosaccharide chains of endogenous tuber glycoproteins. Immunological detection of phosphotyrosylated tuber proteins using anti-(P-Tyr) IgG (Kamps and Sefton, 1988) was performed as referenced above, except that the blocking buffer contained 5% (w/v) BSA (fraction V) and 1% (w/v) ovalbumin instead of 3% (w/v) defatted milk powder. Antigenic polypeptides were visualized using an alkaline-phosphatase-tagged secondary antibody as previously described (Moorhead and Plaxton, 1990). Immunological specificities were confirmed by performing immunoblots in which rabbit preimmune serum was substituted for the affinity-purified IgGs. An LKB Ultrosan XL enhanced laser densitometer was used to scan indicated immunoblots. Densitometric data were analyzed using the LKB Gelscan XL software (version 2.1). Immunoreactive polypeptides were quantified in terms of their relative A_{633} .

Dephosphorylation of Potato Tuber Phosphotyrosyl Proteins by Potato APase

Peeled and diced potato tuber tissue (5 g) was homogenized with a Polytron in 1 volume of 100 mM Mes/NaOH (pH 5.8) containing 2 mM PMSF, 2 mM DTT, and 1 mM EDTA. The homogenate was centrifuged for 10 min at 16,000g in an Eppendorf microfuge, and a 3-mL aliquot of the supernatant fluid was passed at 4°C through a Bio-Rad Econo-Pac 10DG

desalting column that had been pre-equilibrated in homogenization buffer lacking PMSF. Aliquots (0.1 mL) of the desalted crude extract were incubated at 25°C with and without 3 units of the purified potato APase. Samples were removed at 0, 2, and 19 h and analyzed by immunoblotting using the anti-(P-Tyr) IgG as described above. Laser densitometric scanning and immunoquantification of the blots was performed as described above.

Protein Determination

Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad prepared reagent and bovine γ -globulin as standard.

RESULTS

Purification and Physical Properties of Potato Tuber APase

Table I summarizes the purification of potato tuber APase. Only a single peak of APase activity was recovered following chromatography on columns of S-Sepharose, phosphocellulose, and Phenyl Superose. The enzyme was purified 2289-fold to a final PEP-hydrolyzing specific activity of 618 units/mg and an overall recovery of 31%. With P-Tyr as substrate the final specific activity was increased to approximately 1900 units/mg (Table II).

Physical and Immunological Properties

Gel Electrophoresis and Native Molecular Mass Estimation

Nondenaturing PAGE of the final APase preparation resulted in a single protein-staining band that co-migrated with APase activity (Fig. 1a). When the final preparation was denatured and subjected to SDS-PAGE, two major protein-staining bands of 57 and 55 kD were observed (Fig. 2a). Laser densitometric scanning of the SDS gel revealed that the 57- and 55-kD silver stained polypeptides were present in a ratio of about 2:1, respectively. SDS-PAGE of a commercially available preparation of potato APase (Sigma, catalog No. P0157) gave rise to a very similar banding pattern, as was observed for the APase purified in the present study (results not shown). The 57- and 55-kD polypeptides were detected by periodic acid-Schiff staining, indicating that they are glycosylated (Fig. 2b). If the single protein-staining band present after nondenaturing PAGE (Fig. 1a) was excised, equilibrated with SDS, and subjected to SDS-PAGE, both the

Table I. Purification of APase from potato tubers (600 g)

Step	Volume	Activity ^a	Protein	Specific Activity	Purification	Yield
	<i>mL</i>	<i>units</i>	<i>mg</i>	<i>units/mg</i>	<i>fold</i>	<i>%</i>
Crude extract	730	992	3650	0.27		100
Acid step (pH 4.0)	765	975	2715	0.36	1.3	98
PEG fractionation (5–18%)	120	1010	1800	0.56	2.1	102
S-Sepharose	45	525	34	15.4	57	53
Phosphocellulose	11.2	301	1.1	274	1015	30
Phenyl Superose	6.4	309	0.5	618	2289	31

^a Determined using assay A with PEP as substrate.

Table II. Substrate specificity of potato APase

All parameters were determined using assay B as described in "Materials and Methods."

Substrate	V_{max}	K_m	Specificity Constant
	units/mg	mM	V_{max}/K_m
P-Tyr	1917	0.99	1936
p-Nitrophenyl-P	1250	1.10	1136
P-Ser	389	0.62	627
PEP	764	1.52	503
PPi	1728	4.42	391
MgATP	368	2.16	170
P-Thr	99	ND ^a	ND
MgADP	46	ND	ND

^a ND, Not determined.

57- and 55-kD polypeptides were resolved and stained in a ratio of about 2:1, respectively (Fig. 1b).

Determination of the potato APase's native molecular mass was initially attempted by gel filtration FPLC of the final preparation on a calibrated, prepacked Superose 12 HR 10/30 column. Upon gel filtration, however, four peaks of APase activity with estimated molecular masses of 377, 165, 16, and 3 kD were recovered. Following SDS-PAGE of each activity peak, both of the 57- and 55-kD polypeptides were observed and stained in a 2:1 ratio, respectively (results not shown). However, when the purified APase was subjected to nonde-

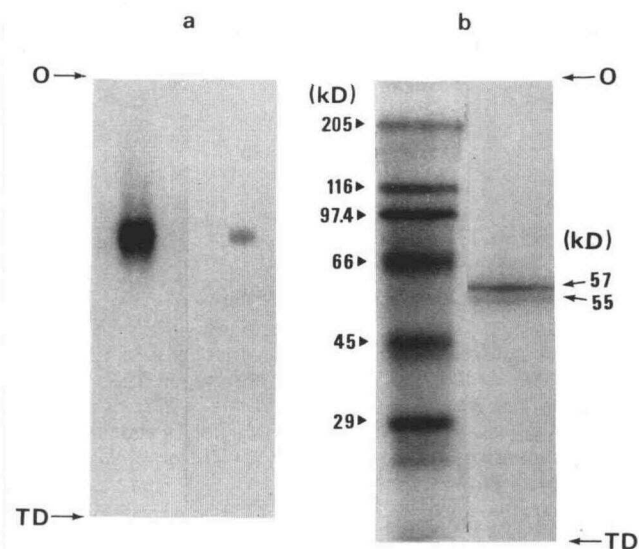


Figure 1. Nondenaturing PAGE of purified potato tuber APase. a, The left lane contained 0.3 µg of protein and was overlaid with an APase activity stain; the right lane contained 1 µg of protein and was stained with Coomassie blue R-250. b, SDS-PAGE of the protein-stained band shown in (a), which had been excised from the nondenaturing gel and equilibrated with SDS as described in "Materials and Methods" (right lane). The polyacrylamide gel concentration of the separating gel was 10% (w/v). SDS-PAGE was conducted according to Laemmli (1970) and the gel was stained with Coomassie blue R-250. O, Origin; TD, tracking dye front.

naturing SDS-PAGE, a single major band of APase activity with a molecular mass of approximately 100 kD was obtained (Fig. 2c).

Cyanogen Bromide Peptide Mapping

The structural relationship between the 57- and 55-kD polypeptides was investigated by peptide mapping of their cyanogen bromide cleavage fragments. As shown in Figure 3, the cleavage patterns of the two polypeptides were similar but not identical. This indicates that the two polypeptides are distinct proteins that may have large regions of similar amino acid sequences.

Heat Stability

The APase was relatively heat stable, losing 0, 52, and 90% of its original activity when incubated for 4 min at 65, 70, and 75°C, respectively.

Immunological Characterization

An immunoblot of the final APase preparation that had been pretreated with sodium *m*-periodate was probed with affinity-purified rabbit anti-(black mustard PEP-specific APase) IgG (Duff et al., 1991) and revealed 57- and 55-kD

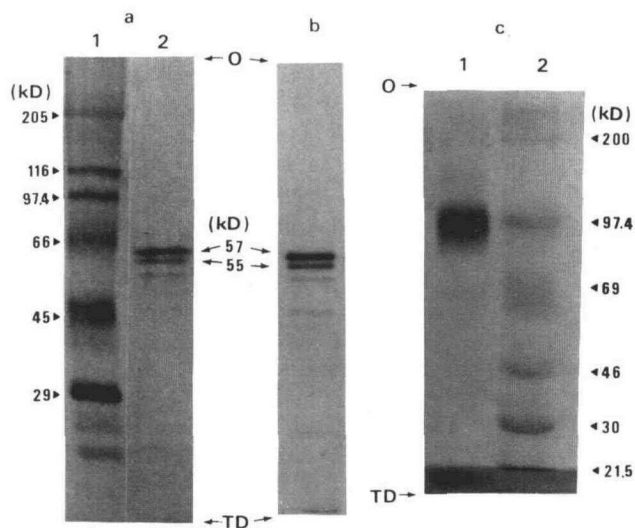


Figure 2. SDS-PAGE of purified potato tuber APase. a and b, Denaturing SDS-PAGE was conducted using a separating polyacrylamide gel concentration of 10% (w/v) according to the method of Laemmli (1970). a, Lane 1 contains 5 µg of molecular mass standards, whereas lane 2 contains 2 µg of the final APase preparation. The gel was stained with silver according to the method of Hochstrasser et al. (1988). b, The single lane contains 2 µg of the final APase preparation and was stained for carbohydrate according to the periodic acid-Schiff silver-staining procedure of Dubray and Bezar (1982). c, Nondenaturing SDS-PAGE was conducted as previously described (Goldstein et al., 1988) using a separating polyacrylamide gel concentration of 8% (w/v). Lane 1 contains 1 µg of the final APase preparation and was overlaid with an APase activity stain; lane 2 contains 5 µg of Amersham prestained "rainbow" molecular mass standards. O, Origin; TD, tracking dye front.

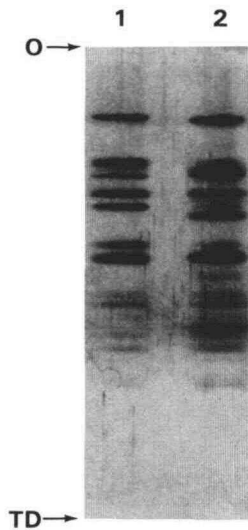


Figure 3. Electrophoretic patterns of cyanogen bromide cleavage fragments of the 57- and 55-kD subunits of potato tuber APase. Cyanogen bromide cleavage fragments were prepared from gel slices containing 5 μg of the 57-kD (lane 1) and 55-kD (lane 2) polypeptides and were analyzed on a 14% (w/v) SDS mini-gel as previously described (Plaxton and Moorhead, 1989). The gel was stained with silver according to the method of Hochstrasser et al. (1988). O, Origin; TD, tracking dye front.

immunoreactive polypeptides that stained in a >2:1 ratio (Fig. 4, lane 1). The same result was obtained after immunoblotting of a clarified potato extract that had been prepared in the absence of protease inhibitors and incubated at 25°C for 0 and 16 h (Fig. 4, lanes 2 and 3, respectively). Identical antigenic polypeptides were observed on immunoblots of clarified potato extracts prepared in the presence of 12 different protease inhibitors or in hot (90°C) SDS-PAGE sample buffer (Fig. 4, lanes 4 and 5, respectively). These data demonstrate that (a) the 55-kD polypeptide observed after SDS-PAGE or immunoblotting of the final APase preparation was not a proteolytic degradation product of the 57-kD protein, and (b) both polypeptides are stable in the absence of added protease inhibitors and are structurally related to the PEP-specific APase from black mustard suspension cells. Figure 4, lane 6, also demonstrates that the amounts and ratio of the 57- and 55-kD proteins remain constant in sprouting (soft) tubers that had been stored for several months at room temperature. The same antibodies have previously been demonstrated to effectively immunoprecipitate the activity of a commercially available preparation of potato tuber APase (Duff et al., 1991).

Kinetic Studies

Unless otherwise stated, all kinetic studies of the potato APase were performed with PEP using assay A.

Effect of pH

The enzyme showed a fairly broad pH/activity profile, with a maximum occurring at about pH 5.8. Half-maximal

activity was observed at pH 4.9 and 6.7. All subsequent kinetic studies were carried out at pH 5.8.

Effect of Divalent Cations

The APase was activated approximately 40% in the presence of saturating (4 mM) Mg^{2+} (added as MgCl_2). Ca^{2+} , Co^{2+} , and Mn^{2+} were individually tested as Cl^- salts at 4 mM, with no added Mg^{2+} , and found to uniformly activate the enzyme by about 30%. When the reaction mixture contained 5 mM EDTA and no added Mg^{2+} , the activity was reduced by about 65%. When tested in place of added Mg^{2+} , 5 mM ZnCl_2

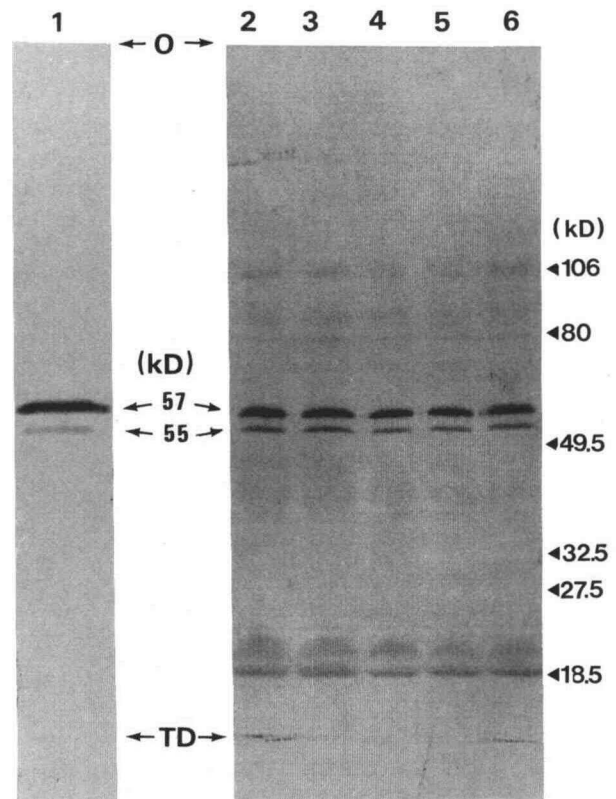


Figure 4. Immunological detection of potato tuber APase. Samples were subjected to SDS-PAGE using a separating polyacrylamide gel concentration of 10% (w/v) according to the method of Laemmli (1970) and the gel was blot-transferred to a polyvinylidene difluoride membrane. The blot was probed with affinity-purified rabbit anti-(black mustard PEP-specific APase) IgG (Duff et al., 1991) and antigenic polypeptides were visualized using an alkaline-phosphatase-linked secondary antibody as described in "Materials and Methods"; alkaline phosphatase staining was for 10 min at 30°C. Lane 1 contains 0.1 μg of the final APase preparation, whereas all other lanes contain 25 μg of protein of a clarified potato extract. Lanes 2 and 3 contain an extract prepared in the absence of protease inhibitors and incubated at 25°C for 0 and 16 h, respectively. Lanes 4 and 5 contain an extract prepared in the presence of 12 different protease inhibitors or in 90°C SDS-PAGE sample buffer, respectively. Lane 6 contains an extract prepared from sprouting (soft) potato tuber tissue. The molecular mass scale is based on the mobilities of the Bio-Rad "low range" prestained SDS-PAGE protein standards. O, Origin; TD, tracking dye front.

Table III. Effect of various substances on the activity of potato APase

The standard assay A was used, except that the concentration of PEP was subsaturating (0.25 mM). Enzyme activity in the presence of effectors is expressed relative to the control set at 100%.

Addition	Concentration	Relative Activity
	<i>mM</i>	<i>%</i>
Vanadate	0.05	7
	0.005	40
Molybdate	1	0
	0.01	5
	0.001	19
EGTA	5	72
EDTA	5	32
NaPi	0.5	44
NaF	5	56
Glutamate	5	30
Aspartate	5	64
Ascorbate	5	12
Phytate	0.1	48
GSH	5	131
Phosvitin	0.001	19

completely inhibited APase activity. All subsequent kinetic studies were conducted in the presence of 4 mM MgCl₂.

Substrate Specificity

APase activity was determined using assay B and a wide range of compounds, tested at a total concentration of 5 mM unless otherwise specified. The purified enzyme showed no activity with AMP, Glc-1-P, or phytate.

Table II lists V_{max} and apparent K_m values, along with specificity constants (V_{max}/K_m), for those compounds that were found to be dephosphorylated at a significant rate by the purified enzyme. The highest activity and specificity constant were obtained with P-Tyr. The specificity constant with P-Tyr was about 3-fold greater than the value obtained with the next best nonartificial substrate, P-Ser. When the egg yolk storage phosphoprotein phosvitin was tested at a concentration of 50 μ g/mL (125 nM), a phosphoprotein phosphatase activity of 220 units/mg was obtained.

Metabolite and Ion Effects

A wide variety of substances was tested for effects on the purified enzyme using subsaturating concentrations of PEP (0.25 mM) as substrate. Table III lists those compounds that were found to inhibit APase activity. The most notable inhibitors were vanadate, molybdate, phytate, phosvitin, and NaPi. The following substances had no effect ($\pm 15\%$ control activity) on enzyme activity. NaCl, KCl, NH₄Cl, Mg-citrate, and tartrate (all 5 mM); cAMP (100 μ M); Ca²⁺/bovine calmodulin (50 μ M/1 μ M); and okadaic acid (1 μ M). Preincubation of enzyme with 1 mM *N*-ethylmaleimide for up to 2 min at 25°C also had no effect on enzyme activity. GSH (5 mM) activated APase activity by 30%.

Dephosphorylation of Potato Tuber Phosphotyrosyl Proteins by Potato APase

Monospecific anti-(P-Tyr) polyclonal antibodies (Kamps and Sefton, 1988) were used to identify proteins phosphorylated on Tyr in potato tuber extracts. The immunoblot shown in Figure 5, lane 1, demonstrates that numerous potato tuber polypeptides appear to be phosphotyrosylated. No antigenic polypeptides were observed when an immunoblot of a potato extract was probed with the P-Tyr antibodies in the presence of 20 mM P-Tyr (Fig. 5, lane 2). By contrast, no reduction in the intensity of the various immunoreactive polypeptides occurred when parallel immunoblots were probed with the anti-(P-Tyr) IgG in the presence of 20 mM P-Ser or P-Thr (results not shown). These control experiments demonstrate the specificity of the polyclonal antibodies against P-Tyr. A very similar banding pattern was observed on an immunoblot of a tuber extract (a) prepared in 90°C SDS-PAGE sample buffer or (b) probed with two commercially available preparations of anti-(P-Tyr) monoclonal antibodies (Sigma, catalog No. P3300, and ICN Biomedicals [Costa Mesa, CA], catalog No. 152369) (results not shown).

When an aliquot of a desalted tuber extract (endogenous APase activity = 0.56 units/mL using assay A) was incubated at 25°C for 19 h, the cross-reactivity of five anti-(P-Tyr) IgG immunoreactive polypeptides was significantly reduced

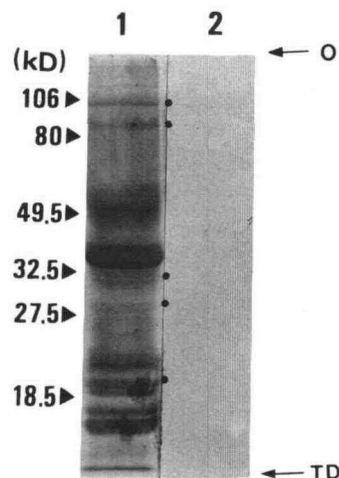


Figure 5. Immunological detection of proteins phosphorylated on Tyr in mature potato tubers. Samples were subjected to SDS-PAGE using a separating gel concentration of 12% (w/v) according to the method of Laemmli (1970) and the gel was blot-transferred to a polyvinylidene difluoride membrane. Lanes 1 and 2 contain 25 μ g of protein from the clarified tuber extract. Lane 1 was probed using affinity-purified rabbit anti-(P-Tyr) IgG (Kamps and Sefton, 1988) and antigenic polypeptides were visualized using an alkaline-phosphatase-linked secondary antibody as described in "Materials and Methods"; phosphatase staining was for 10 min at 30°C. The 102-, 85-, 34-, 28.5-, and 20-kD immunoreactive polypeptides listed in Table IV are indicated (●). Lane 2 was probed as above except 20 mM P-Tyr was added to the anti-(P-Tyr) IgG solution. The molecular mass scale is based on the mobilities of the Bio-Rad "low range" prestained SDS-PAGE protein standards. O, Origin; TD, tracking dye front.

(Table IV). Addition of 30 units/mL of purified potato APase to the desalted tuber extract appeared to markedly enhance the rate and extent of dephosphorylation of the 102-, 85-, and 34-kD phosphotyrosylated polypeptides (Table IV). Silver staining of duplicate SDS gels showed that no reduction in the relative amount of the various polypeptides occurred over the 19-h time course (results not shown). Furthermore, no reduction in the intensity of any of the anti-(P-Tyr) IgG immunoreactive bands occurred when the desalted tuber extract was incubated for 19 h in the combined presence of 1 mM *o*-vanadate and 30 units/mL of the purified potato APase (results not shown).

DISCUSSION

The predominant APase from potato tuber has been purified 2289-fold to near electrophoretic homogeneity and a final P-Tyr-hydrolyzing specific activity of 1917 units/mg. To the best of our knowledge, this final specific activity is the highest yet reported for a purified plant APase (Duff et al., 1994). The native molecular mass of the purified enzyme was estimated by nonreducing SDS-PAGE to be approximately 100 kD (Fig. 2c). A single protein-staining band that co-migrated with APase activity was observed following nonreducing PAGE of the final preparation (Fig. 1a). However, when the final preparation was denatured and subjected to SDS-PAGE, two protein-staining bands with molecular masses of about 57 and 55 kD were observed (Fig. 2a). Densitometric scanning indicated that the 57- and 55-kD protein-staining bands occurred in a ratio of approximately 2:1, respectively. We believe that both polypeptides were associated with native potato APase for the following reasons: (a) these two protein-staining bands co-eluted after Superose 12 gel filtration FPLC of the final preparation; (b) the single band from nonreducing PAGE still produced two bands after SDS-PAGE (Fig. 1b); (c) the respective patterns of peptide fragments produced after limited treatment with cyanogen bromide are quite comparable, as analyzed by SDS-PAGE (Fig. 3); and (d) both polypeptides are im-

munologically related (Fig. 4). These data suggest that the native enzyme could exist as either a homo- or heterodimer.

Immunoblots of potato tuber extracts prepared under conditions permissive and restrictive to endogenous proteolysis demonstrated that the 55-kD polypeptide is not an artifact created by the proteolytic cleavage of the 57-kD subunit after tissue extraction (Fig. 4). Both polypeptides stained strongly as glycoproteins (Fig. 2b), suggesting that their difference in molecular mass could arise from varying degrees of glycosylation. This could explain the problems encountered during Superose 12 FPLC of the final preparation, since the anomalous behavior of a secreted yeast APase during gel filtration chromatography has been attributed to glycosylation (Mildner, 1976). The tendency of phosphatases to aggregate occurs not only with the yeast multigene family of APases (Mildner, 1976) but also with the glycogen phosphorylase phosphatases of skeletal muscle and liver (Rogers et al., 1982). Variation in the extent of glycosylation of a single gene product may be associated with the regulation or targeting of protein phosphatases to fulfill distinct roles (Ballou and Fischer, 1986). Further studies are required to clarify whether the limited heterogeneity of the two potato APase subunits arises as a result of the expression of separate independent genes or from a single gene by differential posttranslational modification.

Zhao and co-workers (1992) recently reported that the dephosphorylation of P-Tyr by potato APase occurs at a rate comparable to that of several human P-Tyr protein phosphatases. This finding is in accord with the relatively high activity and specificity of potato APase for P-Tyr that was observed in the present study (Table II), a property that distinguishes it from most other plant APases that have been examined to date (Duff et al., 1994). The capacity of potato APase to dephosphorylate a variety of phosphoproteins in this and other studies (Bingham et al., 1976; Chen and Bienis, 1990) suggests that such an activity may have physiological relevance. Ser is the predominant phosphorylated amino acid of the egg yolk storage protein, phosvitin (Byrne et al., 1984), and other phosphoproteins that have been reported to serve

Table IV. Dephosphorylation of potato tuber phosphotyrosyl proteins by potato tuber APase

A desalted potato tuber extract was incubated at 25°C in the absence (–) and presence (+) of 30 units/mL purified potato tuber APase. Aliquots were removed at 2 and 19 h and analyzed by immunoblotting using anti-(P-Tyr) IgG (Kamps and Sefton, 1988). The relative P-Tyr content of the five antigenic polypeptides noted in Figure 5 was estimated by laser densitometric quantification of the immunoblots as described in "Materials and Methods." Unless otherwise indicated, all values represent the means ± SE of triplicate determinations.

Estimated Molecular Mass of Phosphotyrosyl Polypeptide	Relative Phosphotyrosyl Content after Incubation at 25°C			
	2 h		19 h	
	–	+	–	+
kD	% initial value			
102	76 ^a	60 ± 5	51 ± 5	36 ± 3
85	85 ± 9	69 ± 2	60 ± 4	34 ± 2
34	93 ± 6	40 ± 3	38 ^a	16 ± 2
28.5	81 ^a	65 ± 2	31 ± 5	28 ± 3
20	83 ^a	66 ± 5	18 ± 1	12 ± 3

^a Mean of duplicate determinations.

as substrates for potato APase (Bingham et al., 1976; Chen and Blenis, 1990). We have shown, however, that similar to other plant tissues (Elliot and Geytenbeek, 1985), there is an abundance of P-Tyr-containing polypeptides in potato tubers (Fig. 5). Moreover, incubation of a desalted tuber extract in the presence of the purified potato APase caused a substantial reduction in the level of phosphorylation of several of the endogenous P-Tyr proteins (Table IV). Since this decrease in protein P-Tyr content was not caused by protein degradation and was negated by the presence of 1 mM *o*-vanadate, these observations suggest that the major potato APase can dephosphorylate endogenous P-Tyr proteins in vitro. It is also important to note that several recent studies have demonstrated that the composition and length of the amino acid sequence adjacent to the phosphotyrosylation site markedly influences the affinity and specificity of a P-Tyr protein phosphatase for its substrate (Cho et al., 1991; Ramachandran et al., 1992; Zhang et al., 1993). For example, a P-Tyr phosphatase from *Escherichia coli* has a K_m (P-Tyr) of more than 6 mM, but displays K_m values ranging from 0.027 to 4.1 mM for various P-Tyr peptides (Cho et al., 1991). Thus, the K_m (P-Tyr) of 0.99 mM derived for potato APase (Table II) may be orders of magnitude in excess of the enzyme's K_m value for P-Tyr peptides and/or endogenous phosphoprotein substrates.

There have been several recent reports describing the purification and characterization of P-Tyr APases from plant sources including poppy seeds (Chung and Polya, 1992), wheat seedlings (Chen and Tao, 1989), and maize seedlings (Jagiello et al., 1992). The subunit composition and substrate specificity of the potato tuber APase most closely resembles that of the poppy seed enzyme (Chung and Polya, 1992). In addition, Polya and Wettenhall (1992) have described the purification and characterization of a minor 28-kD potato tuber APase isoform that also effectively hydrolyzes Pi from P-Tyr. This minor potato APase isoform, however, can be clearly distinguished from the APase of the current study in terms of subunit structure, a P-Tyr-hydrolyzing specific activity of only 103 units/mg, its inability to catalyze dephosphorylation of P-Ser, and its inhibition by micromolar concentrations of cAMP (Polya and Wettenhall, 1992).

Apart from P-Tyr phosphatase activity, the major potato tuber APase isoform shares several properties with various mammalian and yeast P-Tyr protein phosphatases. Similar to the potato APase, the nonplant P-Tyr phosphatases frequently display an acidic pH optimum, show potent inhibition by *o*-vanadate, molybdate, and Zn²⁺, and will catalyze the dephosphorylation of *p*-nitrophenyl-P as well as a variety of other phosphomonoesters (Ballou and Fischer, 1986; Lau et al., 1989; Chen and Blenis, 1990; Cho et al., 1991; Brautigam, 1992; Charbonneau and Tonks, 1992; Pot and Dixon, 1992). Additional studies are necessary to elucidate whether the potato tuber APase's substantial activity with P-Tyr (Table II), and its capacity to dephosphorylate endogenous phosphotyrosylated polypeptides in vitro (Table IV), have any physiological relevance in vivo. Isolation of cDNA clones for potato tuber APase is currently underway and will shed light on the enzyme's relationship with characterized APases and P-Tyr protein phosphatases.

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