Structural and kinetic properties of a novel purple acid phosphatase from phosphate-starved tomato (*Lycopersicon esculentum*) cell cultures

Gale G. BOZZO*, Kashchandra G. RAGHOTHAMA† and William C. PLAXTON*‡¹

*Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6, †Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907-1165, U.S.A., and ‡Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

An intracellular acid phosphatase (IAP) from P_i -starved ($-P_i$) tomato (*Lycopersicon esculentum*) suspension cells has been purified to homogeneity. IAP is a purple acid phosphatase (PAP), as the purified protein was violet in colour ($\lambda_{\max} = 546$ nm) and was insensitive to L-tartrate. PAGE, periodic acid–Schiff staining and peptide mapping demonstrated that the enzyme exists as a 142 kDa heterodimer composed of an equivalent ratio of glycosylated and structurally dissimilar 63 (α -subunit) and 57 kDa (β -subunit) polypeptides. However, the nine N-terminal amino acids of the α - and β -subunits were identical, exhibiting similarity to the deduced N-terminal portions of several putative plant PAPs. Quantification of immunoblots probed with rabbit anti-(tomato acid phosphatase) immune serum revealed that the 4-fold increase in IAP activity due to P_i -deprivation was correlated with similar increases in the amount of antigenic IAP α - and β -subunits. IAP displayed optimal activity at pH 5.1, was

activated 150 % by 10 mM Mg^{2+} , but was potently inhibited by Zn^{2+} , Cu^{2+} , Fe^{3+} , molybdate, vanadate, fluoride and P_i . Although IAP demonstrated broad substrate selectivity, its specificity constant ($V_{\max}/K_m$) with phosphoenolpyruvate was > 250 % greater than that obtained with any other substrate. IAP exhibited significant peroxidase activity, which was optimal at pH 9.0 and insensitive to Mg^{2+} or molybdate. This IAP is proposed to scavenge P_i from intracellular phosphate esters in $-P_i$ tomato. A possible secondary IAP role in the metabolism of reactive oxygen species is discussed. IAP properties are compared with those of two extracellular PAP isoenzymes that are secreted into the medium of $-P_i$ tomato cells [Bozzo, Raghothama and Plaxton (2002) Eur. J. Biochem. 269, 6278–6286].

Key words: acid phosphatase (purple), *Lycopersicon esculentum* (tomato), peroxidase, phosphate starvation (plants).

INTRODUCTION

Acid phosphatases (APs; EC 3.1.3.2) catalyse the hydrolysis of P_i from a broad range of phosphate monoesters and anhydrides with a pH optimum of pH 4–7 [1,2]. APs are ubiquitous and abundant in plants, animals, fungi and bacteria. They function in the production, transport and recycling of P_i , which is crucial for cellular metabolism and bioenergetics. Plant APs are induced under various environmental and developmental conditions, including salt- or water-deficit stress, flowering, fruit ripening, seed germination, senescence and pathogen infection [1,3–5]. AP induction is also a widespread plant response to nutritional P_i -starvation [1,3–7]. P_i is a critical macronutrient that frequently limits plant growth in many natural ecosystems [5–7]. A correlation exists between plant intracellular and secreted AP (IAP and SAP respectively) activity and cellular P_i status [1,5]. SAPs are probably involved in P_i scavenging from extracellular organic phosphate monoesters, whereas IAPs are thought to be pivotal in the remobilization and scavenging of P_i from intracellular phosphate monoesters and anhydrides in P_i -deficient ($-P_i$) plants [3,5,6]. This may be accompanied by P_i transport between different plant tissues.

Purple APs (PAPs) represent a distinct category of non-specific APs containing a binuclear metal-ion complex at their active site [2,8]. They are distinguished from other phosphatases by their characteristic pink or purple colour (due to the presence of a phenolate to metal charge transfer complex), as well as insensi-

tivity to L-tartrate inhibition. Plant PAPs contain a Fe(III)–Zn(II) or Fe(III)–Mn(II) binuclear metal centre, whereas mammalian PAPs have a Fe(III)–Fe(II) unit in their active site [8]. Mammalian PAPs generally occur as 35 kDa monomers, whereas most plant PAPs examined to date exist as 110 kDa homodimers [4,9]. Conserved sequence motifs containing the metal-ligating residues are common to animal and plant PAPs [10,11]. Annotation of the *Arabidopsis* genome predicted 29 different PAP genes, two of which were shown to be responsive to P_i -deprivation [12]. The study by Li et al. [12] demonstrated the complexity and variation of *Arabidopsis* PAP expression and regulation, and implied that numerous PAP isoenzymes probably exist in other plant species. Recent studies indicate that some PAPs are multifunctional proteins that also display alkaline peroxidase activity, hypothesized to function in the metabolism of reactive oxygen species (ROS) during senescence or pathogen infection [3,4].

We recently described the purification and comparison of two P_i -starvation-inducible secreted, but structurally distinct, monomeric PAP isoenzymes (84 kDa SAP1 and 57 kDa SAP2) from the culture medium of heterotrophic $-P_i$ tomato suspension cells [3]. In the present study, we report the purification and characterization of the corresponding IAP from $-P_i$ tomato cells. Our results indicate that the tomato cells markedly up-regulate a single major IAP isoform during P_i stress, and that it exists as an unusual heterodimeric PAP that also displays alkaline peroxidase activity.

Abbreviations used: AP, acid phosphatase; Con A, concanavalin A; DTT, dithiothreitol; IAP, intracellular AP; PAP, purple AP; PEG, poly(ethylene glycol); PEP, phosphoenolpyruvate; $+P_i$ and $-P_i$, P_i -sufficient and P_i -deficient respectively; ROS, reactive oxygen species; SAP, secreted AP.

¹ To whom correspondence should be addressed at the Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6 (e-mail plaxton@biology.queensu.ca).

EXPERIMENTAL

Chemicals and plant material

Fractogel EMD-SO₃⁻-650 (S) cation-exchange resin, glycerol and KCl were from BDH Chemicals. A Superose 12 HR10/30 column, concanavalin A (Con A)–Sephacrose and gel filtration molecular mass standards were from Amersham Biosciences. Acrylamide, bisacrylamide and dithiothreitol (DTT) were from ICN Pharmaceuticals. Ribonucleic acid adjuvant was obtained from RIBI ImmunoChem Research (Hamilton, MT, U.S.A.). PVDF membranes (Immobilon, 0.45 µm pore size) were obtained from Millipore. Alkaline-phosphatase-tagged goat anti-(rabbit IgG) IgG was obtained from Promega. All other chemicals were obtained from Sigma Chemical Co. All solutions were prepared using Milli-Q processed water. Heterotrophic tomato (*Lycopersicon esculentum*, cv. Moneymaker) cell suspensions were kindly provided by Professor Eduardo Blumwald (Department of Pomology, University of California, Davis, CA, U.S.A.), and were routinely cultured in 50 ml of Murashige–Skoog medium containing 2.5 mM P_i (P_i-sufficient, + P_i), as described previously [3]. P_i-deficiency treatments were initiated 7 days after subculturing the cells into the + P_i medium. A 50 ml + P_i cell suspension was centrifuged axenically at 4000 g for 12 min at 25 °C. The cells were washed with – P_i medium, and were used to inoculate 500 ml of – P_i medium. After 8 days, the – P_i cells were harvested by filtration through Whatman 541 filter paper on a Buchner funnel, washed with 10 mM CaCl₂, frozen in liquid N₂ and stored at – 80 °C.

AP assays

All assays were linear with respect to time and concentration of enzyme assayed. One unit of activity is defined as the amount of AP resulting in the hydrolysis of 1 µmol of substrate per min at 25 °C.

Phosphatase assay A

For routine measurements of AP activity, the hydrolysis of phosphoenolpyruvate (PEP) to pyruvate was coupled to the lactate dehydrogenase reaction and was assayed at 25 °C by continuously monitoring the oxidation of NADH at 340 nm using a Gilford 260 recording spectrophotometer. Standard AP assay conditions were 50 mM sodium acetate (pH 5.1), 5 mM PEP, 10 mM MgCl₂, 0.2 mM NADH and 3 units of desalted rabbit muscle lactate dehydrogenase in a final volume of 1 ml. All assays were initiated by the addition of enzyme preparation and corrected for NADH oxidase activity by omitting PEP from the reaction mixture.

Phosphatase assay B

Acid-washed microtitre plates were used for all kinetic studies. For substrates other than PEP, the P_i released by the AP reaction was quantified [13]. Between 1 and 10 m-units of AP (determined using assay A) was incubated in a 96-well microtitre plate in a final volume of 40 µl. Assays contained 50 mM sodium acetate (pH 5.1), 1 mM EDTA, 10 mM MgCl₂ and an alternative substrate (5 mM unless otherwise stated). Assays were initiated by the addition of substrate, allowed to progress at 25 °C for 6 min and terminated by the addition of 200 µl of a solution which was prepared daily by mixing four parts of 10 % (w/v) ascorbate with one part of 10 mM ammonium molybdate in 15 mM zinc acetate (pH 5.0). Samples were incubated for 25 min at 40 °C and the A₆₆₀ determined using a Spectromax 250 Microplate

spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). Controls were run to check background levels of P_i present at each substrate concentration tested. To calculate activities, a standard curve over the range 1–133 nmol of P_i was constructed for each set of assays.

Kinetic studies and protein concentration determination

Apparent $V_{\max}$ and K_m values were calculated from the Michaelis–Menten equation fitted to a non-linear least-squares regression computer kinetics program [14]. Competitive and uncompetitive inhibition constants are represented as K_i and K_i' respectively. K_i values were determined from Dixon plots, whereas K_i' values were determined from Cornish-Bowden plots [15]. All kinetic parameters are the means of three separate experiments and are reproducible within ± 10 % of the mean.

Protein concentrations were determined with the Coomassie Blue G-250 dye-binding method [16] using bovine γ-globulin as the protein standard.

Peroxidase assay

A chemiluminescence assay was employed to assess the capacity of the purified tomato IAP to catalyse the peroxidation of 5-aminophthalhydrazide (luminol) [3]. Chemiluminescence was recorded using a Lmax Microplate Luminometer (Molecular Devices). The reaction was initiated by the addition of 0.1 ml of 4.4 mM H₂O₂ to 0.1 ml of 0.2 M Tris/HCl (pH 7.4) containing 300 mM luminol and 5–20 nM of IAP in a 96-well microtitre plate. Photon emission was measured continuously for 10 s after H₂O₂ addition, and was expressed as relative light units with Softmax data analysis software (Molecular Devices). In control experiments, equimolar concentrations of BSA or horseradish peroxidase (EC 1.11.1.7) were substituted for purified IAP.

Buffers used in the purification of tomato IAP

Buffer A contained 50 mM potassium acetate (pH 5.6), 1 mM EDTA, 1 mM DTT, 1 mM 2,2'-dipyridyl disulphide, 5 mM thio-urea and 1 mM PMSF. Buffer B contained 50 mM sodium acetate (pH 5.0), 1.5 mM MgCl₂, 1 mM EDTA and 1 mM DTT. Buffer C contained 50 mM sodium acetate (pH 5.0), 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 10 % (v/v) glycerol. Buffer D contained 25 mM sodium acetate (pH 5.7), 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.2 mM CaCl₂, 0.2 mM MnCl₂ and 10 % (v/v) glycerol. Buffer E contained 25 mM sodium acetate (pH 5.7), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 1.5 mM MgCl₂ and 10 % (v/v) glycerol.

Purification of tomato IAP

All steps were carried out at 0–4 °C. Quick-frozen – P_i tomato suspension cells (500 g) were ground to a powder in liquid N₂ using a mortar and pestle, homogenized (1:2, w/v) in buffer A with 50 g of acid-washed sand, and centrifuged at 14 000 g for 20 min. Poly(ethylene glycol) 8000 (PEG; 50 % [w/v] dissolved in 20 mM Bistris-propane/HCl [pH 7.4] containing 1 mM EDTA) was added to the supernatant to a final concentration of 3.5 % (w/v). The solution was stirred for 30 min and centrifuged as above. Finely ground PEG 8000 was added to the supernatant fluid to a final concentration of 20 % (w/v). The extract was stirred for 60 min and centrifuged for 20 min at 14 000 g. PEG pellets were resuspended in 100 ml of Buffer B to yield a protein

concentration of approx. 4 mg/ml. The solution was stirred for 20 min, centrifuged as above, and absorbed at 1.5 ml/min onto a column (2.2 cm $\times$ 7.5 cm) of Fractogel EMD-SO₃⁻-650 (S) that had been connected to an ÄKTA FPLC system and pre-equilibrated with Buffer C. The column was washed at 2 ml/min with buffer C until the A_{280} decreased to baseline, and then with buffer C containing 400 mM KCl. The column was developed with a linear gradient (285 ml) of 0.4–1 M KCl in Buffer C (fraction size = 8 ml). A single peak of AP activity eluted at approx. 600 mM KCl. Pooled AP activity fractions were concentrated to 22 ml using an Amicon YM-30 ultrafilter. The concentrate was applied at 0.5 ml/min onto a column (1 cm $\times$ 3.2 cm) of Con A–Sephacrose pre-equilibrated with Buffer D. AP activity was eluted using 70 ml of a linear gradient of 0–500 mM methyl α -D-mannopyranoside in Buffer D (fraction size = 2 ml). Peak activity fractions were pooled, concentrated to 0.15 ml, and applied at 0.2 ml/min onto a Superose 12 HR 10/30 column, which had been connected to the FPLC system and pre-equilibrated with Buffer E (fraction size = 0.5 ml). Peak activity fractions were pooled and concentrated with a Centricon-30 to 0.55 ml, divided into 15 μ l aliquots, frozen in liquid N₂, and stored at -80°C . The purified IAP was stable for at least 4 months when frozen.

Antibody production

Purified IAP (150 μ g) was dialysed overnight against PBS, filtered through a 0.2 μ m membrane, and emulsified in Ribi adjuvant (1 ml total volume). After collection of pre-immune serum, the AP was injected (0.6 ml subcutaneously, 0.4 ml intramuscularly) into a 2 kg New Zealand rabbit. A secondary injection (80 μ g) was administered subcutaneously after 28 days. At 10 days after the final injection, blood was collected by cardiac puncture. After incubation overnight at 4°C , the clotted cells were removed by centrifugation at 1000 g for 10 min. The antiserum was frozen in liquid N₂ and stored at -80°C in 0.04 % (w/v) NaN₃.

Electrophoresis and immunoblotting

SDS/PAGE was performed with a Bio-Rad Minigel apparatus as described previously [17]. Subunit molecular masses were calculated from a plot of relative mobility against log [molecular mass (kDa)], with the following protein standards: myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase B (97.5 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Carbohydrate staining was performed using a periodic acid–Schiff staining procedure [3].

Non-denaturing PAGE was conducted at 4°C as described previously [17]. To detect in-gel AP activity, a gel was incubated for 1 h at 25°C in 100 mM sodium acetate (pH 5.3), followed by incubation in the same buffer containing 10 mM MgCl₂, 1 mg/ml Fast Garnet GBC salt and 0.03 % (w/v) β -naphthyl-phosphate [3]. To estimate native molecular mass by non-denaturing PAGE, a plot of log ($R_f \times 100$) against the percentage gel concentration yielded a straight line, the negative slope of which is the retardation coefficient. A log–log plot of the retardation coefficient against the molecular mass (kDa) of the calibration proteins produced a linear curve from which the native molecular mass of IAP was estimated [18]. The calibration proteins used for the construction of the Ferguson plot were the non-denatured protein molecular mass standards (Sigma): bovine milk α -lactalbumin (14.2 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), chicken ovalbumin (45 kDa), BSA (monomer, 66 kDa;

dimer, 132 kDa) and jack bean urease (trimer, 272 kDa; hexamer, 545 kDa).

Immunoblotting was performed by transferring proteins from SDS gels on to PVDF membranes by electroblotting for 75 min at 100 V [17]. Before immunoblotting, carbohydrate epitopes were abolished by a sodium-*m*-periodate oxidation procedure as described previously [19]. Antigenic polypeptides were visualized using an alkaline-phosphatase-tagged secondary antibody [20]. The relative amount of IAP protein in clarified extracts from 8-day-old $+P_i$ compared with $-P_i$ tomato cells was determined by quantification of the antigenic α - and β -subunits of IAP (see the Results section) on immunoblots (in terms of A_{663}) using an LKB Ultrosan XL laser densitometer and GelScan software (Version 2.1) (Amersham Biosciences). Derived A_{663} values were linear with respect to the amount of the immunoblotted extract. Immunological specificities were confirmed by performing immunoblots in which rabbit pre-immune serum was substituted for the anti-(tomato IAP) immune serum.

Peptide mapping by CNBr cleavage

Polypeptides corresponding to the α - and β -subunits of purified IAP were excised from SDS/PAGE minigels and cleaved *in situ* with CNBr as described previously [21]. The degradation products were analysed on an SDS/PAGE 14 % minigel, followed by staining with SYPRO Red (Amersham Biosciences). Fluorescence imaging was performed using a Typhoon 9400 Imager Workstation (Amersham Biosciences).

Amino acid sequencing

N-terminal and tryptic peptide sequencing were performed by automated Edman degradation at the Laboratory for Macromolecular Structure (Purdue University). Similarity searches were conducted with the BLAST program using the 'short but nearly exact' option available on the National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov/>).

RESULTS

Influence of P_i -starvation on growth and AP activity of tomato suspension cells

Tomato suspension cells cultured for 8 days in the absence of exogenous P_i had 40 % of the fresh mass of the 8-day-old $+P_i$ cells (approx. 63 and 25 g of cells were obtained per 500 ml culture of 8-day-old $+P_i$ and $-P_i$ cells respectively). At 8 days following subculture of the tomato cells into $-P_i$ culture medium, IAP activity increased from approx. 0.4 to 1.6 units/mg protein (but remained unchanged in $+P_i$ cells). IAP specific activity decreased to 0.4 units/mg within 2 days of an 8-day-old $-P_i$ cell culture being resupplied with 2.5 mM P_i . All subsequent studies were performed using 8-day-old $-P_i$ cells.

IAP purification from $-P_i$ tomato suspension cells

A single AP activity peak was resolved during all chromatographic steps. As outlined in Table 1, IAP from the $-P_i$ tomato cells was purified approx. 476-fold to a final PEP-hydrolysing specific activity of 620 units/mg protein, and a recovery of approx. 11 %. Binding of the enzyme to immobilized Con A suggested that this IAP is glycosylated. IAP in the concentrated Con A–Sephacrose and Superose 12 fractions was violet in colour. The physical basis for the violet colour of IAP is apparent from its

Table 1 Purification of IAP from 500 g of P_i -starved tomato suspension cells

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Clarified extract	1000	2000	2672	1.3	—	—
PEG fractionation	115	437	1541	3.5	2.7	58
SO ₃ ⁻ Fractogel	96	14.8	1062	72	55	40
Con A-Sepharose	11	1.7	789	464	357	30
Superose 12 FPLC	0.55*	0.49	304	620	476	11

* Concentrated pooled fractions.

Figure 1 Visible absorbance spectrum of tomato IAP

The spectrum was obtained using a solution containing 12.5 mg/ml of IAP in buffer D (see the Experimental section).

visible absorption spectrum (Figure 1), which shows that the protein absorbs maximally in the middle of the visible region (peaking at 546 nm).

Physical and immunological properties of tomato IAP

Gel electrophoresis and immunoblotting

Non-denaturing PAGE of the final IAP preparation resulted in a single protein-staining band that co-migrated with AP activity

(Figure 2A). When the purified IAP preparation was denatured and subjected to SDS/PAGE, two major protein staining bands of 63 (α -subunit) and 57 kDa (β -subunit) were resolved (Figure 2B). Laser densitometric scanning of the SDS gel revealed that both polypeptides stained with Coomassie Blue with equal intensities. When the single protein-staining band present after non-denaturing PAGE (Figure 2A) was excised, equilibrated with SDS, and subjected to SDS/PAGE, 63 and 57 kDa polypeptides were resolved, which stained for protein and cross-reacted with rabbit anti-(tomato IAP) immune serum with equivalent intensities (results not shown). IAP's α - and β -subunits were both detected by periodic acid-Schiff staining, confirming that they are glycosylated (Figure 2C).

Immunoblotting with rabbit anti-(tomato IAP) immune serum was used to estimate the relative amount of IAP in clarified extracts from the 8-day-old $+P_i$ compared with $-P_i$ tomato cells. In each instance, immunoreactive 63 and 57 kDa polypeptides were observed (Figure 2D, lanes 2 and 3) that co-migrated with the α - and β -subunits of purified IAP (Figure 2D, lane 1). Laser densitometric quantification of the immunoblots revealed that the $-P_i$ extracts contained approx. 3-fold more of the IAP α - and β -subunits relative to extracts of $+P_i$ cells. Equal intensity staining 63 and 57 kDa antigenic polypeptides were also observed on an immunoblot of a clarified $-P_i$ tomato cell extract that was prepared under denaturing conditions in the presence of 10% (w/v) trichloroacetic acid [22] (results not shown).

Immunoblotting was also employed to assess the immunological relatedness of tomato IAP with the two secreted PAP isoenzymes (i.e. 84 kDa monomeric SAP1; 57 kDa monomeric

Figure 2 PAGE and immunoblot analyses of purified IAP from $-P_i$ tomato suspension cell cultures

(A) Non-denaturing PAGE (7% separating gel) analysis of purified IAP. Lanes 1 and 2 contain 2 μ g and 1 μ g of the final IAP preparation respectively. Lane 1 was stained with Coomassie Blue R-250, whereas lane 2 was subjected to an in-gel AP activity stain [3]. (B) SDS/PAGE analysis (10% separating gel) of 1 μ g of purified IAP. Protein staining was performed with Coomassie Blue R-250. (C) Carbohydrate staining of 5 μ g of purified IAP resolved by SDS/PAGE. Glycoprotein staining was performed using a periodic acid-Schiff procedure [3]. (D) Immunoblot analysis was performed using 2500-fold diluted rabbit anti-(tomato IAP) immune serum. Antigenic polypeptides were visualized using an alkaline-phosphatase-tagged secondary antibody. Lane 1 contains 10 ng of purified IAP. Lanes 2 and 3 contain 4 μ g of protein from a clarified extract of 8-day-old $+P_i$ and $-P_i$ cells respectively. Lane 4 contains 50 ng of homogeneous tomato SAP1, whereas lane 5 contains 25 ng of purified tomato SAP2 [3].

Table 2 Comparison of the N-terminal (a) and tryptic peptide (b) sequences of the 63 kDa α -subunit and 57 kDa β -subunit of tomato IAP with the deduced amino acid sequence for several plant PAPs

The N-terminal and tryptic peptide sequences of tomato IAP's α - and β -subunits were determined by automated Edman degradation. Other sequences were deduced by translation of plant PAP nucleotide sequences. Sequence numbering represents the amino acid position relative to the N-terminus. Dashes denote the amino acid residues that are identical with those of the respective tomato IAP sequences. An asterisk denotes an unknown amino acid residue, and the underlined sequence indicates that no significant similarity with other protein sequences was identified.

(a)		
	Sequence	GenBank® GI
<i>L. esculentum</i> 63 kDa α -subunit	1 T*FPSVDIP 9	
<i>L. esculentum</i> 57 kDa β -subunit	1 TQFPSVDIP 9	
<i>Allium cepa</i> PEP phosphatase	37 SE--A---- 45	14349285
<i>Oryza sativa</i> secretory PAP precursor	31 -EY--T--- 39	29466964
<i>O. sativa</i> PAP	31 -EY--T--- 39	19879917
<i>Lupinus luteus</i> PAP	34 SE---T--- 41	21464654
(b)		
	Sequence	GenBank® GI
<i>L. esculentum</i> 57 kDa β -subunit	APIYITVGG	
<i>A. cepa</i> PEP phosphatase	384 --V-----D 392	14349285
<i>Nicotiana tabacum</i> PAP	382 --V-----D 390	27597227
<i>O. sativa</i> secretory PAP precursor	378 --V-----D 386	29466964
<i>Arabidopsis thaliana</i> calcineurin-like phosphoesterase	379 --V-----D 387	15242870
<i>N. tabacum</i> PAP	387 -----I-D 395	27597239
<i>A. thaliana</i> PAP11	362 -----I-D 370	15224112
<i>A. thaliana</i> calcineurin-like phosphoesterase	381 -----I-D 389	15234369
<i>A. thaliana</i> Fe(III)-Zn(III) PAP precursor	386 -----I-D 394	15225855
<i>Anchusa officinalis</i> PAP	387 -----I-D 395	11262830
<i>A. thaliana</i> calcineurin-like phosphoesterase	381 -----I-D 389	15223530
<i>A. thaliana</i> secreted PAP precursor	386 -----I-D 394	1218042
<i>L. esculentum</i> 63 kDa α -subunit	<u>DDTNNI</u>	

SAP2) that accumulate in the culture medium of $-P_i$ tomato cells [3]. The anti-(tomato IAP) immune serum cross-reacted with an immunoblot of 25 ng of purified SAP2 (Figure 2D, lane 5). In contrast, the same antibody preparation failed to detect 50 ng of purified SAP1 (Figure 2D, lane 4).

Determination of native molecular mass

This was attempted by gel filtration FPLC of the enzyme on a calibrated Superose 12 HR10/30 column. Upon gel filtration, however, a single peak of AP activity eluting with an apparent molecular mass of 50 kDa was recovered. Following SDS/PAGE of the gel filtration AP activity peak, 63 and 57 kDa polypeptides were resolved and stained for protein in a 1:1 ratio, respectively (Figure 2B). Electrophoresis of the native enzyme in non-denaturing gels of varying acrylamide concentrations consistently resolved a single protein staining band (Figure 2A), and by Ferguson plot analysis [18], yielded a M_r of 142000 ± 9000 ($n = 3$). Thus the native IAP appears to be heterodimeric.

Peptide mapping and amino acid sequencing

The structural relationship between the IAP α - and β -subunits was investigated by peptide mapping of their CNBr-cleavage fragments. The CNBr-cleavage patterns of the two polypeptides were dissimilar (Figure 3).

The nine N-terminal amino acids of IAP's α - and β -subunits were sequenced by automated Edman degradation and were determined to be identical (Table 2). BLAST analysis revealed significant similarity to a portion of the deduced N-terminal region of several plant PAPs (Table 2). A nine-amino-acid sequence of an α -subunit tryptic peptide exhibited significant similarity with

Figure 3 Electrophoretic patterns of CNBr-cleavage fragments of the 63 kDa α -subunit and 57 kDa β -subunit of tomato IAP

Gel slices containing 1 μ g each of non-digested β -subunit (lane 1) and α -subunit (lane 2), and CNBr-cleavage fragments prepared from gel slices containing 4 μ g each of β -subunit (lane 3) and α -subunit (lane 4) were analysed on an SDS/14% PAGE minigel as previously described [20]. Peptides were stained with SYPRO Red and image analysis was performed using a Typhoon imaging workstation. The migration of various molecular mass standards is shown on the left.

a portion of the conserved phosphohydrolase domain of various plant PAPs (Table 2). Conversely, a six-amino-acid sequence of a β -subunit tryptic peptide did not show significant similarity to any protein in the NCBI database.

Figure 4 Dependence of various IAP kinetic parameters on assay pH

(A) $V_{\max}/K_m$ and $\log V_{\max}$; (B) K_m and pK_m . Data were obtained by varying the concentration of PEP. Assays were buffered by a mixture of 25 mM sodium acetate and 25 mM Mes. All values are the means of three independent determinations and are reproducible to within $\pm 10\%$ of the mean.

Heat stability

The enzyme was relatively heat-labile, losing 20, 50 and 80 % of its original activity when incubated for 5 min at 50, 65 and 70 °C respectively.

IAP kinetic properties

Unless otherwise stated, all kinetic studies were performed using assay A. IAP showed a relatively narrow pH phosphatase-activity profile, with maximal activity and specificity constants ($V_{\max}/K_m$) for PEP occurring at about pH 5.1 (Figure 4A). The apparent K_m (PEP) values were minimal and equivalent (approx. 0.2 mM) in the range pH 4.9–5.4 (Figure 4B). The slope of the descending part of the pK_m curve in Figure 4B (approx. 0.8) suggests that the change in the protonation state of one species, either in the free enzyme or of PEP, is responsible for the corresponding change in affinity [23]. The corresponding pK_a of such a group is approx. pH 5.8. The pK_a values corresponding to the protonation equilibria of the enzyme–substrate complex ($pK_{es1} = 4.9$, $pK_{es2} = 5.7$) are derived from the $\log V_{\max}$ against pH plot (Figure 4A).

Effect of bivalent metal cations

Tomato IAP was activated by approx. 150 % and 130 % in the presence of 10 mM $MgCl_2$ and 10 mM $MnCl_2$ respectively. There was no effect on IAP activity when the reaction mixture contained $BaCl_2$, $CaCl_2$, $CoCl_2$, KCl , $NaCl$ or $EDTA$ (10 mM each). Conversely, the IAP was potently inhibited by $CuSO_4$, $ZnCl_2$ and $FeCl_3$ (10 mM each), which caused 88 %, 99 % and 100 % inhibition of IAP activity respectively.

Substrate specificity

IAP activity was determined using assay B and a wide range of phosphorylated compounds, tested at a concentration of 5 mM un-

Table 3 Substrate saturation kinetics of IAP

Kinetic parameters were determined using assay B. All values are the means of at least three independent determinations and are reproducible to within $\pm 10\%$ of the mean.

Substrate	$V_{\max}$ (units/mg)	K_m (mM)	$V_{\max}/K_m$ (units/mg per mM)
PEP	676	0.22	3072
ATP	558	0.50	1116
Bis-(<i>p</i> -nitrophenyl)phosphate*	1323	1.4	945
β -Naphthyl phosphate*	147	0.18	817
IDP	157	0.23	682
GTP	96	0.16	600
Phosphotyrosine	1622	4.1	396
ADP	353	1.4	252
PP_i	610	3.4	179
UTP	289	2.5	116
α -Naphthyl-phosphate*	349	3.4	103
GDP	246	3.6	68
Phenyl-phosphate	390	7.1	55
Pyridoxal 5-phosphate	126	2.7	46

* Synthetic substrate.

less otherwise specified. The purified enzyme showed no activity when tested with dihydroxyacetone phosphate, cAMP, ribose 5-phosphate, ribulose 1,5-bisphosphate, ADP–glucose, UDP–glucose or phytate, and no phosphodiesterase activity was detected with 5 mM bis-(*p*-nitrophenyl)phosphate. Low IAP activity (10–20 % relative to that observed with 5 mM PEP) was observed with AMP, CMP, UMP, mannose 6-phosphate, 3-phosphoglycerate, glucose 1-phosphate, glucose 6-phosphate, phosphoserine and phosphothreonine.

Kinetic parameters of IAP for those compounds that were identified as being the most effective substrates are listed in Table 3. In general, the enzyme appears to be relatively non-specific. Maximal specificity constants ($V_{\max}/K_m$) for physiologically relevant substrates were obtained with PEP, ATP, IDP and GTP. IAP's apparent $V_{\max}$ and K_m value for PEP were equivalent when phosphatase assay A was used in place of assay B. Hyperbolic saturation kinetics were observed in all cases.

Metabolite and ion effects

A wide variety of compounds were examined for effects on IAP activity with subsaturating concentrations of PEP (1.5 mM). The following compounds exerted no influence ($\pm 10\%$ control velocity) on IAP activity: L-tartrate, L-glutamate, L-aspartate, D-asparagine, isocitrate, succinate, glycolate, formate, glyoxylate, phosphite and H_2O_2 (10 mM each). Significant inhibition was exerted by molybdate, vanadate, P_i and fluoride (Table 4). Inhibition by these compounds was characterized further, and the patterns of inhibition and inhibition constants are presented in Table 4. Inhibition of IAP activity by molybdate was competitive, whereas fluoride, vanadate and P_i showed a mixed pattern of inhibition. Increased assay pH was correlated with increases in the inhibition constants (K_i' and/or K_i values) for molybdate, fluoride and vanadate, but a reduction in these values for P_i . Inhibition patterns were unaffected by assay pH.

Peroxidase activity of tomato IAP

The ability of tomato IAP to catalyse the peroxidation of luminol was investigated using a chemiluminescence assay. In the presence of luminol and H_2O_2 , the purified IAP induced striking luminescence when compared with a BSA control. Photon emission induced by IAP peroxidase activity was proportional to enzyme concentration. IAP displayed a relatively sharp

Table 4 Influence of assay pH on the inhibition pattern and enzyme-inhibitor dissociation constants for several inhibitors of tomato IAP

Patterns of inhibition and K_i and K_i' values (representing competitive and uncompetitive inhibition constants respectively) were determined using assay A as described in the Experimental section. All values are the means of three independent determinations and are reproducible to within $\pm 10\%$ of the mean.

Inhibitor	Assay pH	Inhibition type	K_i (mM)	K_i' (mM)
Molybdate	5.1	Competitive	0.0002	—
	5.7	Competitive	0.0004	—
	6.2	Competitive	0.0005	—
Fluoride	5.1	Mixed	0.094	0.25
	5.7	Mixed	0.14	0.96
	6.2	Mixed	0.85	1.2
Vanadate	5.1	Mixed	0.0065	0.014
	5.7	Mixed	0.013	0.075
	6.2	Mixed	0.015	0.12
P_i	5.1	Mixed	1.6	6.3
	5.7	Mixed	0.42	0.75
	6.2	Mixed	0.14	0.32

pH/peroxidase activity profile in the alkaline range, with maximal activity occurring at approx. pH 9.0 (results not shown). Calibration of the luminometer with known amounts of horseradish peroxidase (116 units/mg of protein) allowed for the estimation of a specific peroxidase activity of about 10 units/mg of protein for IAP at pH 9.0. Molybdate, $ZnCl_2$ or P_i (5 mM each) exerted no influence on the peroxidase activity of the purified IAP.

DISCUSSION

At 8 days following subculture of heterotrophic tomato suspension cells into $-P_i$ medium, IAP specific activity increased by approx. 4-fold (but remained low and constant in $+P_i$ cells). Quantification of immunoblots probed with anti-(tomato IAP) immune serum indicated that the induction of tomato IAP activity by P_i deprivation was correlated with a similar increase in the amount of total antigenic IAP protein. This implies that *de novo* IAP synthesis occurred during P_i -starvation of the tomato cell culture. *De novo* synthesis of APs in response to P_i -starvation has been reported for several plants, including *Brassica nigra* and *Brassica napus* suspension cells [1,24,25].

The bis-(*p*-nitrophenyl)phosphate-hydrolysing specific activity (1323 units/mg) of the final IAP preparation compares favourably with the values reported for a range of homogeneous plant APs (Table 5) [1,26,27]. The native molecular mass of purified IAP was

determined by non-denaturing PAGE to be approx. 142 kDa. A single protein-staining band that co-migrated with AP activity was observed following non-denaturing PAGE of the final preparation (Figure 2A). However, when purified IAP was denatured and subjected to SDS/PAGE and immunoblotting, equal intensity staining 63 (α -subunit) and 57 kDa (β -subunit) polypeptides were resolved that strongly cross-reacted with the anti-(tomato IAP) immune serum (Figures 2B and 2C). We believe that both polypeptides were associated with native tomato IAP, since the single protein-staining band from non-denaturing PAGE produced the 63 and 57 kDa polypeptides following second-dimension SDS/PAGE. Furthermore, the 63 and 57 kDa polypeptides co-purified in a 1:1 ratio during cation-exchange, Con A-Sepharose and Superose 12 gel filtration FPLC.

Analysis of the N-terminal amino acid sequence of tomato IAP's α - and β -subunits produced the same sequence, which was maximally similar to onion (*Allium cepa*), rice (*Oryza sativa*) and lupin (*Lupinus luteus*) PAPs (Table 2). These enzymes belong to a plant PAP subfamily, distinguished by the absence of a cysteine residue around position 350 of the polypeptide sequence [28]. A tryptic peptide sequence of IAP's 57 kDa α -subunit was highly similar to a portion of the conserved phosphohydrolase domain present in the C-terminal region of several plant PAPs and calcineurin-like phosphoesterases (Table 2). Similar to tomato IAP, onion PAP was purified as a 240 kDa tetramer consisting of an equivalent ratio of 52 and 42 kDa polypeptides [29]. However, the 42 kDa polypeptide of onion PAP was hypothesized to have arisen via proteolytic cleavage of the 52 kDa polypeptide [29]. In the present study, the detection of the immunoreactive 63 and 57 kDa IAP polypeptides following trichloroacetic-acid extraction of $-P_i$ tomato suspension cells indicates that the 57 kDa β -subunit was not a proteolytic degradation product of the 63 kDa α -subunit. This was corroborated by highly dissimilar CNBr peptide maps of IAP's α - and β -subunits (Figure 3). CNBr-fragmentation patterns depend on the position and number of methionine residues in the protein [21]. Overall, our results indicate that a single IAP is induced by $-P_i$ tomato suspension cells and that it exists as an unusual 142 kDa heterodimer composed of equal proportions of structurally dissimilar α (63 kDa) and β (57 kDa) subunits that share at least some regions of sequence similarity.

Tomato IAP is a PAP since it exhibited a λ_{max} at 546 nm (Figure 1) and a violet colour in solution, and was insensitive to L-tartrate inhibition. The majority of plant PAPs that have been studied are homodimers of 55 kDa subunits [8,9], whereas tomato SAP1 and SAP2 are monomeric PAP isoenzymes (Table 5) [3]. To our knowledge, tomato IAP is the first heterodimeric PAP to be characterized. The enzyme is presumably localized in

Table 5 Comparison of selected physical and kinetic characteristics of intracellular (IAP) with secreted (SAP) purple acid phosphatase isoenzymes from $-P_i$ tomato suspension cell cultures

All physical and kinetic data for SAP1 and SAP2 are taken from Bozzo et al. [3].

Property	IAP	Secreted acid phosphatases	
		SAP1	SAP2
Subunit structure	142 kDa heterodimer	84 kDa monomer	57 kDa monomer
Absorption maximum (nm)	546	518	538
Glycosylated	Yes	Yes	Yes
Activity remaining after 5 min at 70 °C (%)	20	100	100
V_{max} with pNPP (units/mg)	1323	246	940
Specificity constant (V_{max}/K_m) with PEP (units/mg per mM)	3022	115	274
Phosphate ester substrate selectivity	Non-specific	Non-specific	Non-specific
Phosphatase pH optima	5.1	5.3	5.3
Peroxidase pH optima	9.0	8.4	8.4

the cell vacuole, a location which is consistent with the IAP's acidic pH optima (Figure 4), and for which AP activity is a well-characterized biochemical marker. Subcellular fractionation studies demonstrated that the P_i -starvation-inducible IAP from *B. nigra* suspension cells is vacuolar [1].

Baldwin et al. [30] isolated and characterized a novel tomato P_i -starvation-induced gene (*LePS2*) encoding a 30 kDa protein having 40% sequence similarity with a chicken phosphatase. Bacterially expressed recombinant LePS2 showed low phosphatase activity with bis-(*p*-nitrophenyl)phosphate at acidic pH. LePS2 was suggested to function as a P_i -regulated AP to enhance the availability and utilization of organic P_i in $-P_i$ tomato [30]. However, the results of the present and previous [3] studies do not support LePS2 functioning as a predominant non-specific AP in $-P_i$ tomato. Recent findings indicate that *LePS2* actually encodes a P_i -starvation inducible phosphoprotein phosphatase (J. C. Baldwin and K. G. Raghothama, unpublished work).

Binding of IAP to Con A–Sephrose and its subsequent elution with methyl α -D-mannopyranoside indicated that the enzyme was glycosylated. This was confirmed by periodic acid–Schiff staining of IAP's α - and β -subunits (Figure 2D), and provides a rationale for the anomalous molecular mass (50 kDa) estimated for IAP during FPLC of the native enzyme on a calibrated Superose 12 column. The unusual behaviour of potato tuber and yeast APs during gel filtration chromatography has been attributed to glycosylation [27,31].

The 57 kDa SAP2, but not 84 kDa SAP1 from culture medium of the $-P_i$ tomato cells [3] cross-reacted strongly with the anti-(tomato IAP)-immune serum (Figure 2D). This implies that tomato IAP and SAP2 share common antigenic determinants, but that SAP1 is quite distinct. Likewise, the IAP from yam tubers was demonstrated to be immunologically related to one of two yam SAP isoenzymes [32].

Kinetic properties of tomato IAP

IAP was activated by Mg^{2+} and Mn^{2+} , and was potently inhibited by Zn^{2+} and Cu^{2+} . Analogous results have been obtained with various plant APs [1,26,27], including the P_i -starvation-inducible SAP1 and SAP2 from culture medium of $-P_i$ tomato suspension cells [3]. Similar to other APs [1], IAP was subject to potent competitive inhibition by molybdate, whereas mixed inhibition was exerted by vanadate, fluoride and P_i (Table 4). Molybdate and vanadate serve as a transition-state analogue of the pentavalent-phosphate-reaction intermediate [33]. The mode of fluoride inhibition of bovine spleen PAP was shown to pH-dependent [34]. The pattern of IAP inhibition by molybdate, vanadate, fluoride and P_i was independent of pH changes in the range of 5.1–6.2 (Table 4). Nevertheless, with the exception of P_i , an increased K_i' and/or K_i value was correlated with a pH increase from pH 5.1 to 6.2. The mixed pattern of inhibition by the product P_i (Table 4) was also reported for the 84 kDa monomeric SAP1 from $-P_i$ tomato [3]. Conversely, many plant APs, including tomato SAP2, are competitively inhibited by P_i [1]. The different modes of P_i inhibition may arise from differences in structural and/or conformational properties of APs [35]. Inhibition by P_i is in accord with our hypothesis that tomato IAP plays an important P_i -recycling role during P_i -starvation, when cellular P_i levels may be decreased by up to 50-fold [5].

In general, most PAPs characterized to date are considered to be non-specific APs [10]. Conversely, a PAP purified from germinating soybeans exhibited significant specificity for phytic acid [36]. A portion of a cDNA encoding the active-centre residues of a diphosphonucleotide phosphatase/phosphodiesterase from

yellow lupin was similar to the metal-ligating residues of PAPs [37]. However, tomato IAP is neither a phytase nor a PAP with phosphodiesterase activity, since it displayed no activity with phytic acid or bis-(*p*-nitrophenyl)phosphate. The maximal V_{max} (1622 units/mg) of tomato IAP was obtained with *O*-phospho-L-tyrosine (Table 3), comparable with a non-specific potato tuber IAP exhibiting significant phosphotyrosine phosphatase activity [27]. Tomato IAP was more efficient at hydrolysing P_i from physiological substrates with good leaving groups (Table 3), whereas those with poorer leaving groups ($\Delta G^{\circ} < 5$ kcal/mol) [38] were not efficiently used. This phenomenon was also apparent for both SAP isoenzymes from $-P_i$ tomato [3]. However, the specificity constants (V_{max}/K_m) of the tomato IAP for similarly tested substrates were 1–2 orders of magnitude greater than those obtained with either of the two SAPs from culture medium of the $-P_i$ tomato cells [3]. Thus tomato IAP exhibits higher substrate-utilization efficiency relative to the corresponding SAP isoenzymes.

In terms of apparent K_m and specificity constant, PEP was the most effective substrate for tomato IAP (Table 3). The tomato IAP displayed a relatively low K_m (PEP) value of approx. 0.22 mM (Table 3), which compares with K_m (PEP) values of 0.44 and 0.05 mM, respectively, reported for onion bulb PAP and a P_i -starvation-inducible IAP of *B. nigra* cell cultures [29,39]. The K_m (PEP) of *B. nigra* IAP is in the range of PEP concentrations reported for the plant cytoplasm (i.e. approx. 25–100 μ M) [39]. *B. nigra* IAP was hypothesized to function during P_i -starvation as a PEP phosphatase to bypass the ADP-limited pyruvate kinase while recycling valuable P_i [25]. A range of phosphorylated metabolites was also effectively hydrolysed by tomato IAP, indicating that it should be designated as a non-specific AP. Hence tomato IAP may play a pivotal role during P_i -deficient growth to scavenge and remobilize intracellular P_i from phosphate monoesters and anhydrides. Studies correlating the pattern of IAP expression with *in vivo* levels of intracellular phosphate metabolites in response to P_i nutritional status will help to elucidate the physiological role of IAP. Comparative analyses of SAP1 and SAP2 expression should define a complex framework for the expression and physiological relevance of all three P_i -starvation-inducible tomato PAP isoenzymes.

The pH optimum of 5.1 determined for tomato IAP is in the range of previously characterized PAPs (Table 5) [1,2]. The pK_a of 5.8 estimated from the pK_m (PEP) against pH profile (Figure 4B) may be due to the presence of an ionizable group in the enzyme, since it is significantly different from PEP's pK_a values ($pK_{a1}' \leq 2.0$, $pK_{a2}' = 3.5$ and $pK_{a3}' = 6.4$) [40]. In studies of the yam PAP active site, a pK_a of 4.7 was attributed to protonation of an aquo ligand co-ordinated at Fe(III) of the Fe(III)–Zn(II) centre [41]. The active-site centre of tomato IAP and the mechanism of phosphate ester hydrolysis remain to be assessed, but may be similar to those determined for other plant PAPs.

PAPs displaying both AP activity and alkaline peroxidase activity have been reported for tomato, *Arabidopsis* and recombinant human enzymes [3,4,42]. Tomato IAP also displayed alkaline peroxidase activity that was insensitive to potent AP inhibitors. This suggests that the tomato IAP is a bifunctional protein, as was reported for both secreted tomato PAPs [3]. Plant PAPs displaying peroxidation activity have been hypothesized to function in the generation of ROS, which is closely associated with the oxidative burst that occurs during the plant defence response to pathogen infection [3,4]. An *Arabidopsis* PAP displaying peroxidase activity that is up-regulated during P_i -starvation was suggested to play an additional role in ROS metabolism during senescence [4]. The generation of intracellular ROS is associated with an increase in respiratory electron transport and O_2 uptake

during biotic and abiotic stresses, and is followed by programmed cell death in *Arabidopsis* [43]. Peroxidase activity of tomato IAP might be associated with the metabolism of intracellular ROS following loss of vacuolar integrity, which occurs during programmed cell death [44]. IAP activity may also be pivotal for mobilizing intracellular P_i from senescing tissue in tomato. Future studies are required to determine whether IAP is induced in senescing or pathogen-treated tomato cells, and whether the peroxidase activity of IAP plays an intermediary role between vacuolar autolysis and cellular degenerative processes in response to abiotic or biotic oxidative stresses.

Concluding remarks

Together with our earlier study [3], the current work completes the purification and biochemical characterization of the principal P_i -starvation-inducible AP isoenzymes of tomato suspension cells. It is notable that the single IAP and two SAP isoenzymes that are up-regulated by $-P_i$ tomato cells are all PAPs that exhibit alkaline peroxidase activity (Table 5). P_i -starvation-inducible extracellular PAPs have been demonstrated to occur in duckweed and *Arabidopsis* [4,45], whereas Northern blotting revealed that two putative *Arabidopsis* PAPs are induced by P_i deprivation [12]. To the best of our knowledge, however, the present study provides the first evidence that a plant intracellular PAP is also P_i -starvation-inducible. Isolation of cDNA clones encoding tomato IAP's α - and β -subunits, as well as tomato SAP1 and SAP2, is currently underway. This should help to shed light on the genetic origin, structural relatedness, control of expression and respective functions of the three P_i -starvation-inducible tomato PAP isoenzymes.

This work was supported by research and equipment grants from the Natural Sciences and Engineering Research Council of Canada (to W. C. P.), and U.S. Department of Agriculture National Research Initiative Competitive Grants Program to (K. G. R.). We are grateful to Dr William Turner for helpful discussions.

REFERENCES

- Duff, S. M. G., Sarath, G. and Plaxton, W. C. (1994) The role of acid phosphatases in plant phosphorus metabolism. *Physiol. Plant.* **90**, 791–800
- Vincent, J. B., Crowder, M. W. and Averill, B. A. (1992) Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. *Trends Biochem. Sci.* **17**, 105–110
- Bozzo, G. G., Raghothama, K. G. and Plaxton, W. C. (2002) Purification and characterization of two secreted purple acid phosphatase isozymes from phosphate-starved tomato (*Lycopersicon esculentum*) cell cultures. *Eur. J. Biochem.* **269**, 6278–6286
- del Pozo, J. C., Allona, I., Rubio, V., Leyva, A., de la Peña, A., Aragoncillo, C. and Paz-Ares, J. (1999) A type 5 acid phosphatase gene from *Arabidopsis thaliana* is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. *Plant J.* **19**, 579–589
- Plaxton, W. C. and Carswell, M. C. (1999) Metabolic aspects of the phosphate starvation response in plants. In *Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization* (Lerner, H. R., ed.), pp. 349–372. Marcel Dekker, New York
- Raghothama, K. G. (1999) Phosphate acquisition. *Annu. Rev. Plant Physiol. Mol. Biol.* **50**, 665–693
- Vance, C. P., Uhde-Stone, C. and Allan, D. L. (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytol.* **157**, 423–447
- Schenk, G., Ge, Y., Carrington, L. E., Wynne, C. J., Searle, I. R., Carrol, B. J., Hamilton, S. and de Jersey, J. (1999) Binuclear metal centers in plant purple acid phosphatases: Fe–Mn in sweet potato and Fe–Zn in soybean. *Arch. Biochem. Biophys.* **370**, 183–189
- LeBansky, B. R., McKnight, T. D. and Griffing, L. R. (1992) Purification and characterization of a secreted purple acid phosphatase from soybean suspension cells. *Plant Physiol.* **99**, 391–395
- Schenk, G., Guddat, L. W., Ge, Y., Carrington, L. E., Hume, D. A., Hamilton, S. and de Jersey, J. (2000) Identification of mammalian-like purple acid phosphatases in a wide range of plants. *Gene* **250**, 117–125
- Klabunde, T., Sträter, N., Krebs, B. and Witzel, H. (1995) Structural relationship between the mammalian Fe(III)–Fe(II) and the Fe(III)–Zn(II) plant purple acid phosphatase. *FEBS Lett.* **367**, 56–60
- Li, D., Zhu, H., Liu, K., Liu, X., Leggewie, G., Udvardi, M. and Wang, D. (2002) Purple acid phosphatases of *Arabidopsis thaliana*: comparative analysis and differential regulation by phosphate deprivation. *J. Biol. Chem.* **277**, 27772–27781
- Drueckes, P., Schinzel, R. and Palm, D. (1995) Photometric microtiter assay of inorganic phosphate in the presence of acid-labile organic phosphates. *Anal. Biochem.* **230**, 173–177
- Brooks, S. P. G. (1992) A simple computer program with statistical tests for the analysis of enzyme kinetics. *Biotechniques* **13**, 906–911
- Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, Butterworth, London
- Bollag, D. M., Rozycki, M. D. and Edelstein, S. J. (1996) *Protein Methods*, pp 62–70, Wiley-Liss, New York
- Moraes, T. F. and Plaxton, W. C. (2000) Purification and characterization of phosphoenolpyruvate carboxylase from *Brassica napus* (rapeseed) suspension cell cultures: implications for phosphoenolpyruvate carboxylase regulation during phosphate starvation, and the integration of glycolysis with nitrogen assimilation. *Eur. J. Biochem.* **267**, 4465–4476
- Chrambach, A. and Rodbard, D. (1971) Polyacrylamide gel electrophoresis. *Science* **172**, 440–451
- Laine, A. C. (1988) Significant immunological cross-reactivity of plant glycoproteins. *Electrophoresis* **9**, 841–844
- Moorhead, G. B. G. and Plaxton, W. C. (1990) Purification and characterization of cytosolic aldolase from carrot storage root. *Biochem. J.* **269**, 133–139
- Plaxton, W. C. and Moorhead, G. B. G. (1989) Peptide mapping by CNBr fragmentation using a sodium dodecyl sulfate-polyacrylamide mini-gel system. *Anal. Biochem.* **178**, 391–393
- Wu, F. S. and Wang, M. Y. (1984) Extraction of proteins for sodium dodecyl sulfate-polyacrylamide gel electrophoresis from protease-rich plant tissues. *Anal. Biochem.* **139**, 100–103
- Dixon, M. and Webb, E. C. (1979) *Enzymes* (3rd edn), Longman, London
- Duff, S. M. G., Plaxton, W. C. and Lefebvre, D. D. (1991) Phosphate-starvation response in plant cells: *de novo* synthesis and degradation of acid phosphatases. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9537–9542
- Carswell, M. C., Grant, B. R. and Plaxton, W. C. (1997) Disruption of the phosphate-starvation response of oilseed rape suspension cells by the fungicide phosphonate. *Planta* **203**, 67–74
- Turner, W. L. and Plaxton, W. C. (2001) Purification and characterization of banana fruit acid phosphatase. *Planta* **214**, 243–249
- Gellatly, K. S., Moorhead, G. B. G., Duff, S. M. G., Lefebvre, D. D. and Plaxton, W. C. (1994) Purification and characterization of a potato tuber acid phosphatase having significant phosphotyrosine phosphatase activity. *Plant Physiol.* **106**, 223–232
- Olczak, M. and Wątor, W. (2003) Two subfamilies of plant purple acid phosphatases. *Physiol. Plant.* **118**, 491–498
- Shinano, T., Yonetani, R., Ushihara, N., Adachi, H., Wasaki, J., Matsui, H. and Oaski, M. (2001) Characteristics of phosphoenolpyruvate phosphatase purified from *Allium cepa*. *Plant Sci.* **161**, 861–869
- Baldwin, J. C., Karthikeyan, A. S. and Raghothama, K. G. (2001) *LEPS2*, a phosphorus starvation-induced novel acid phosphatase from tomato. *Plant Physiol.* **125**, 728–737
- Mildner, P. (1976) Purification of protoplast-secreted acid phosphatase from baker's yeast. *Biochim. Biophys. Acta* **429**, 274–282
- Kamenan, A., Diopoh, J. and Colas, B. (1985) Immunological relatedness of cytoplasmic and membrane-bound phosphatases from *Dioscorea rotunda*. *Plant Sci. Lett.* **39**, 83–87
- Lindqvist, Y., Schneider, G. and Vikho, P. (1994) Crystal structures of rat acid phosphatase complexed with transition-state analogs vanadate and molybdate: implications for the reaction mechanism. *Eur. J. Biochem.* **221**, 139–142
- Pinske, M. J., Merckx, M. and Averill, B. A. (1999) Fluoride inhibition of bovine spleen purple acid phosphatase: characterization of a ternary enzyme–phosphate–fluoride complex as a model for the active enzyme–substrate–hydroxide complex. *Biochemistry* **38**, 9926–9936
- Vincent, J. B., Crowder, M. W. and Averill, B. A. (1992) Multiple binding sites for tetrahedral oxoanion inhibitors of bovine spleen purple acid phosphatase. *Biochemistry* **31**, 3033–3037
- Hegeman, C. E. and Grabau, E. A. (2001) A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. *Plant Physiol.* **126**, 1598–1608

- 37 Olczak, M. and Olczak, T. (2002) Diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus* L.) belongs to a novel group of specific metallophosphatases. *FEBS Lett.* **519**, 159–163
- 38 Jencks, W. P. (1976) Free energies of hydrolysis and decarboxylation. In *CRC Handbook of Biochemistry and Molecular Biology: Physical and Chemical Data* (Fasman, G. D., ed.), vol. 1, 3rd edn, p. 302, CRC Press, Cleveland
- 39 Duff, S. M. G., Lefebvre, D. D. and Plaxton, W. C. (1989) Purification and characterization of a phosphoenolpyruvate phosphatase from *Brassica nigra* suspension cells. *Plant Physiol.* **90**, 734–741
- 40 Wold, F. and Ballou, C. E. (1957) Studies on the enzyme enolase: I. Equilibrium studies. *J. Biol. Chem.* **227**, 301–312
- 41 Durmus, A., Eicken, C., Sift, B. H., Kratel, A., Kappl, R., Hüttermann, J. and Krebs, B. (1999) The active site of purple acid phosphatase from sweet potatoes (*Ipomoea batatas*). *Eur. J. Biochem.* **260**, 709–716
- 42 Kaija, H., Alatalo, S. L., Halleen, J. M., Lindqvist, Y., Schneider, G., Väänänen, H. K. and Vihko, P. (2002) Phosphatase and oxygen radical-generating activities of mammalian purple acid phosphatase are functionally independent. *Biochem. Biophys. Res. Comm.* **292**, 128–132
- 43 Tiwari, B. S., Belenghi, B. and Levine, A. (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant. Physiol.* **128**, 1271–1281
- 44 Thomas, H., Ougham, H. J., Wagstaff, C. and Stead, A. D. (2003) Defining senescence and death. *J. Exp. Bot.* **54**, 1127–1132
- 45 Nakazato, H., Okamoto, T., Nishikoori, M., Washio, K., Morita, N., Haraguchi, K., Thompson, Jr, G. A. and Okuyama, H. (1998) The glycosylphosphatidylinositol-anchored phosphatase from *Spirodela oligorrhiza* is a purple acid phosphatase. *Plant Physiol.* **118**, 1015–1020

Received 25 June 2003/26 August 2003; accepted 1 October 2003

Published as BJ Immediate Publication 1 October 2003, DOI 10.1042/BJ20030947