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Binuclear Metal Centers in Plant Purple Acid Phosphatases: Fe–Mn in Sweet Potato and Fe–Zn in Soybean[1]

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Purple acid phosphatases comprise a family of binuclear metal-containing acid hydrolases, representatives of which have been found in animals, plants, and fungi. The goal of this study was to characterize purple acid phosphatases from sweet potato tubers and soybean seeds and to establish their relationship with the only well-characterized plant purple acid phosphatase, the FeIII–ZnII-containing red kidney bean enzyme. Metal analysis indicated the presence in the purified sweet potato enzyme of 1.0 g-atom of iron, 0.6–0.7 g-atom of manganese, and small amounts of zinc and copper. The soybean enzyme contained 0.8–0.9 g-atom of iron, 0.7–0.8 g-atom of zinc per subunit, and small amounts of manganese, copper, and magnesium. Both enzymes exhibited visible absorption maxima at 550–560 nm, with molar absorption coefficients of 3200 and 3300 M^{-1} cm^{-1}, respectively, very similar to the red kidney bean enzyme. Substrate specificities were markedly different from those of the red kidney bean enzyme. A cloning strategy was developed based on N-terminal sequences of the sweet potato and soybean enzymes and short sequences around the conserved metal ligands of the mammalian and red kidney bean enzymes. Three sequences were obtained, one from soybean and two from sweet potato. All three showed extensive sequence identity (>66%) with red kidney bean purple acid phosphatase, and all of the metal ligands were conserved. The combined results establish that these enzymes are binuclear metalloenzymes: Fe–Mn in the sweet potato enzyme and Fe–Zn in soybean. The sweet potato enzyme is the first well-defined example of an Fe–Mn binuclear center in a protein.

Key Words: **acid phosphatase; binuclear metalloenzyme; purple acid phosphatase; sweet potato; soybean.**

In plants the assimilation and maintenance of adequate levels of phosphate require the presence of a group of enzymes which release phosphate from phosphate esters and anhydrides (1). Depending on the optimum pH for activity these enzymes are classified as acid or alkaline phosphatases. Detailed characterization of the enzymes is necessary in order to determine their individual biological roles.

Purple acid phosphatases catalyzing the hydrolysis of a broad range of phosphoric acid esters and anhydrides have been characterized from animal, plant, and fungal sources (for a recent review see Klabunde and Krebs (2)). The mammalian enzymes are closely related with respect to size (~35 kDa), amino acid sequence, and metal content. The active form of the enzyme is pink (λ_{max} = 510 nm) and contains a binuclear FeIII–FeII metal center in which the iron atoms are antiferromagnetically coupled. Oxidation converts the enzyme to the inactive, purple FeIII–FeIII form (λ_{max} = 550 nm). Plant purple acid phosphatases have been isolated from red kidney bean (3) and soybean seeds (4, 5), sweet potato tubers (6–8), spinach leaves (5), duckweed (9), and suspension cultures of rice (10) and soybean (11). The well-characterized red kidney bean enzyme is a homodimer, each subunit of ~55 kDa con-

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taining a binuclear FeIII–ZnII center (3). The three-dimensional structure of the enzyme (12) has revealed the amino acid residues which provide the metal ligands, and a comparison of the amino acid sequences of the red kidney bean and animal enzymes shows that these residues are conserved in all of the enzymes, despite overall low sequence homology between them (<20%) (13). Furthermore, substitution of ZnII in the red kidney bean enzyme active site with FeII generates a redox-active form of the enzyme with spectroscopic properties which are very similar to those of the mammalian enzymes (14). A general catalytic mechanism for the purple acid phosphatases, which is consistent with the available structural, spectroscopic, and kinetic data, involves coordination of the phosphate ester substrate by the divalent metal ion, followed by nucleophilic attack by a water molecule coordinated to the ferric ion (12).

While the initial studies on the red kidney bean enzyme, which showed the presence of the binuclear FeIII–ZnII center, were generally uncontroversial, the same has not been true for the other plant enzymes for which quantitative metal analyses are available. Fujimoto *et al.* (6) reported 2 g-atoms of Mn(III) per 100-kDa dimer of sweet potato enzyme (isolated from Kokei cultivar), whereas Sugiura *et al.* (8) found only 1 g-atom of Mn(III) per dimer (enzyme from Kintoki cultivar). In contrast, Hefler and Averill (15) reported 2 g-atom of Fe per dimer of sweet potato enzyme and no Mn and speculated that the iron was present in mononuclear centers (1 per subunit). The presence of ~1 g-atom of manganese per 58-kDa subunit and negligible iron or zinc has been reported for the soybean enzyme (purified from seeds (4) and from suspension culture (11)). The presence of 0.3 g-atoms of Fe and 0.25 g-atoms of Mn in a purple acid phosphatase from duckweed has been reported recently (9), and some evidence for the presence of nonstoichiometric amounts of manganese in the rice (10) and spinach (5) enzymes has also been obtained.

Since the sweet potato and soybean enzymes appear similar in many respects to the red kidney bean enzyme (size, subunit structure, and visible spectra), and since both metal ions of the binuclear metal centre of the red kidney bean and mammalian purple acid phosphatases have been implicated in catalysis, it seems unlikely *a priori* that either the soybean or sweet potato enzyme contains a mononuclear metal center. We have therefore purified and characterized both enzymes in order to reassess their metal ion status. We have also examined their specificities toward a range of activated and unactivated phosphate esters and anhydrides to allow a detailed comparison with the red kidney bean enzyme and comment on possible functions. While the function of the enzyme is unknown, the observation that in some species (e.g., rice, soybean, and duckweed) the activity is phosphate-repressible is consistent with a role in phosphate acquisition (1). In order to check the closeness of the relationship of the sweet potato and soybean enzymes to the red kidney bean, we have cloned and sequenced the corresponding purple acid phosphatase cDNAs. Definition of the metal centers in plant purple acid phosphatases additional to the red kidney bean enzyme, together with their amino acid sequences, will provide a better understanding of the relationship between these proteins.

EXPERIMENTAL

Materials. Soybean seeds (cultivar Provar) and sweet potato tubers (cultivar Golden) were purchased locally. Molecular biology reagents and DNA-modifying enzymes were purchased from New England Biolabs, Promega, Progen Industries, and Boehringer-Mannheim.

Enzyme purification. Sweet potato purple acid phosphatase was purified by a four-step procedure adapted from a literature method (16). Juice was extracted from 60 kg of sweet potato tubers at room temperature. All subsequent steps were performed at 4°C. Acetone precipitation (25–45%) was followed by ammonium sulfate precipitation (35–70%), dialysis against 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA and DEAE-cellulose chromatography in the same buffer. Activity was eluted with a linear salt gradient between 0.25 and 0.35 M NaCl. The eluted enzyme was concentrated by ultrafiltration and further purified by gel filtration using Sephadex G150 superfine (Pharmacia) in 0.1 M acetate buffer, pH 4.90.

Soybean purple acid phosphatase was purified by an adaptation of a literature method (4). Soybeans (3 kg) were soaked overnight in distilled water at room temperature. All subsequent steps were performed at 4°C. The soaked beans were extracted with 10 l of 0.2 M acetic acid. The extract was filtered through cheesecloth and centrifuged. The supernatant was subjected to ammonium sulfate precipitation (30–65%) and the pellet dialyzed against 0.01 M phosphate buffer, pH 6.0. The dialyzed material was applied to CM-cellulose equilibrated with the same buffer and eluted with a linear NaCl gradient (0–0.5 M). The pooled activity was further purified on concanavalin A–Sepharose equilibrated with 0.5 M NaCl. Soybean purple acid phosphatase was eluted with 5% α-D-methylmannoside in 0.5 M NaCl. Eluted fractions were dialyzed against 0.01 M phosphate buffer, pH 6.0, and applied to CM-Trisacryl equilibrated with the same buffer. Enzyme activity eluted in two peaks by a linear 0–0.5 M NaCl gradient. SDS–PAGE analysis indicated that the protein in the second peak was homogenous. The major (earlier eluting) peak was further purified using ultrafiltration and chromatography, first on Sephacryl S-300 equilibrated with 0.5 M NaCl and then on concanavalin A–Sepharose equilibrated with 0.5 M NaCl. Protein was eluted with a linear gradient (0–5%) of α-D-methylmannoside. The purified enzymes were >95% pure as judged by SDS–PAGE (17).

Determination of protein concentration. Concentrations of the purified enzymes were determined from the A_{280}, using values of $A^{1\%}_{1\ cm}$ of 21.4 for soybean purple acid phosphatase (4) and 27.0 for sweet potato purple acid phosphatase (determined in this study using the method of Blakeley and Zerner (18)).

Metal ion analysis. Metal analyses were performed using either inductively coupled plasma–atomic emission spectroscopy (ICP-

TABLE I

Oligonucleotides Used for Cloning of Sweet Potato and Soybean Purple Acid Phosphatases

Primer	Sequence	Location[a]
	Forward	
1	5′-GARGAYGTNGAYATGCCNTGGGAYAG-3′	118–144
2	5′-TGGGAYAGYGAYGTNTTYGCNGTNCC-3′	136–162
3	5′-ACATTTGGTCTAATAGGGGAYCTWGG-3′	484–510
4	5′-CCATGGATATGGACTGCAGGGAAYCAYG-3′	679–707
	Reverse	
1	5′-CCGATCTCTCATAGGCATGAACRTGNCC-3′	1062–1090
2	5′-TTCATGGTTSCCTGCAGTCCADATCCA-3′	681–708
3	5′-CCAAATGTATATGGTACATCARGNCC-3′	465–491
4 (SP-PAP1a)	5′-ATGCAGAGTAGCTTGGCTGTGGCTGTGT-3′	1216–1243
5 (SP-PAP1b)	5′-CAGGTCCAGGTTTGGGAGGAGTAA-3′	449–472
6 (SP-PAP2a)	5′-CTGCTTGCCGCTTTATTGGATTTCTTTC-3′	547–574
7 (SP-PAP2b)	5′-CGTGTTCCCAATCCCAACCTCATAT-3′	406–429
8 (SB-PAPa)	5′-CTGCACAAACCAGGGCTCATACATGAC-3′	1012–1038
9 (SB-PAPb)	5′-TCAGGACCAATTTCAGGAGGAGTCACA-3′	711–737

[a] Numbering according to *A. thaliana* cDNA sequence (20). Residue numbers are lower by 18 for the red kidney bean sequence. SP-PAP1 and SP-PAP2, isoforms 1 and 2 of sweet potato purple acid phosphatase; SB-PAP, soybean purple acid phosphatase. a and b are nested primers for 5′-RACE.

AES)[4] using a Spectra Analytical Instruments Model M+P spectrometer or inductively coupled plasma–mass spectroscopy (ICP-MS) using a Perkin–Elmer SCIEX-ELAN 5000 spectrometer. Samples and standards were prepared in 0.1 M acetate buffer, pH 4.9, for ICP-AES analysis and in 1% HNO_3 for ICP-MS. Separate standard curves were routinely prepared for iron, zinc, copper, and manganese. Errors associated with these analyses were ~±3%. In analyses of the soybean enzyme before and after dialysis external calibration using a multielemental standard was used. Errors associated with these analyses were ~±15%. Metal stoichiometries were expressed as g-atoms per 55-kDa subunit (sweet potato) or per 58-kDa subunit (soybean).

Enzyme assays. Sweet potato purple acid phosphatase activity was determined by a continuous assay using *p*-nitrophenyl phosphate (*p*-NPP) as substrate. To 3.0 ml of 0.1 M acetate buffer, pH 4.90, equilibrated at 25°C, was added an aliquot (0.1 ml) of *p*-NPP (final concentration, 5 mM) followed by an aliquot of enzyme. The change in absorbance at 390 nm was measured ($\Delta\epsilon$ = 342.6 M^{-1} cm^{-1}). Soybean purple acid phosphatase was routinely assayed by a discontinuous assay; 0.1 ml of *p*-NPP (final concentration 3.5 mM) followed by an aliquot of enzyme was added to 1.0 ml of 0.1 M acetate buffer, pH 4.90, 0.5 M NaCl, equilibrated at 30°C. After 5 min the reaction was terminated by the addition of 2.0 ml of 0.1 M NaOH, and the absorbance at 400 nm was measured ($\Delta\epsilon$ = 18320 M^{-1} cm^{-1}). Kinetic constants (k_{cat} and K_m) were determined by addition of an aliquot of substrate solution followed by an aliquot of enzyme to 0.1 M acetate buffer, pH 4.90 at 25°C (for sweet potato purple acid phosphatase) or 0.1 M acetate buffer, pH 5.5 containing 1 mg/ml bovine serum albumin at 30°C (for soybean purple acid phosphatase). At time intervals samples were withdrawn and the phosphate concentration determined as described previously (19). Kinetic constants were determined by nonlinear regression analysis of initial rate data.

[4] Abbreviations used: ICP-AES, inductively coupled plasma–atomic emission spectroscopy; ICP-MS, inductively coupled plasma–mass spectroscopy, *p*-NPP, *p*-nitrophenyl phosphate.

Cloning of sweet potato and soybean purple acid phosphatases. Total RNA from sweet potato tuber and soybean leaf tissues was extracted using the TRIZOL reagent (Gibco BRL) as recommended by the manufacturer. cDNAs were cloned utilising the 5′- and 3′-RACE methods (Boehringer-Mannheim). For 3′-RACE, forward primers 1 and 2 (Table I) were based on the N-terminal amino acid sequence of sweet potato purple acid phosphatase, determined in the present work. A single sequence was obtained. The sequence exhibited extensive similarity with the reported sequence for the soybean enzyme (11):

Sweet potato LPNAED**VDMPWDSDVFA**VPSGYNAPQQVHI
Soybean KVEKA**VDMPLDSDVFA**R

Forward primers 3 and 4 and reverse primers 1–3 were based on alignment of cDNA sequences for red kidney bean (Accession No. AJ001270) and *Arabidopsis thaliana* purple acid phosphatases (20). All except reverse primer 3 encompass one of the conserved motifs around the metal ligands. Reverse primer 3 corresponds to a conserved sequence close to one of the metal-binding motifs. The gene-specific primers used for 5′-RACE were based on sequence information obtained from 3′-RACE (Table I). Primers were synthesized by Pacific Oligos (Lismore, Australia). The same conditions were applied to all PCR reactions unless stated otherwise: a "hotstart" incubation at 94°C for 2 min was followed by 40 cycles of denaturation (94°C, 15 s), annealing (55°C, 15 s), and extension (72°C, 45 s). The product of first-strand cDNA synthesis was used as template for second-strand synthesis with forward primer 1 and reverse anchor primer (supplied in the kit). The 3′-terminal sequence (~800 nucleotides) was amplified from this product using three rounds of semi-nested PCR using reverse anchor primer and successive forward primers 2–4. The 5′-terminal sequence (~400 nucleotides) was amplified similarly using forward primer 1 and successive reverse primers 1–3. The central region (~800 nucleotides) was amplified using forward primer 2 and reverse primer 1. The overlap between the 5′ and central sequences was 355 bp and between the 3′ and central sequences 411 bp. The assembled sequences encoded all but the first 13 (sweet potato purple acid phosphatase) or 12 (soybean purple acid phosphatase) amino acids of the mature proteins and their signal

peptides. Sequence data for these regions were obtained using 5′-RACE.

DNA and protein sequencing and analysis. Automated DNA sequencing applying the dideoxy chain termination method (21) was performed in the Australian Genome Research Facility using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit. The N-terminal sequence of sweet potato purple acid phosphatase was determined by automated Edman degradation using an Applied Biophysics Model 470A Gas-Phase Protein/Peptide Sequencer. Nucleotide and amino acid sequences were aligned using Clustal W software (22).

RESULTS

Purification. Purification of sweet potato purple acid phosphatase from 60 kg of tubers as described under Experimental reproducibly yielded ~100 mg of enzyme, which migrated as a single band on SDS–PAGE. The specific activity of fractions across the final elution profile ranged from 730 to 918 U/mg. The final specific activity is comparable with values previously reported for the purified enzyme (650 U/mg (16) and 751 U/mg (15)), albeit measured under slightly different assay conditions. Purification of the soybean enzyme from 3 kg of beans yielded ~5 mg of enzyme. Two forms of the enzyme which were separated by ion-exchange chromatography had identical specific activities and apparent molecular weights. When the gel filtration step was performed prior to ion exchange, most of the activity eluted in the second peak from the ion-exchange column, suggesting that gel filtration removed some material associated with the enzyme, which caused it to be eluted earlier. All experiments were done using the second peak of activity. The specific activity of the final preparation was 100 U/mg at pH 4.9 and 220 U/mg at pH 5.5. Previous preparations exhibited specific activities of 84 U/mg (pH 5.5, 35°C) (5) and 512 U/mg (pH 5.5, 22°C) (11).

Comparison of molecular and catalytic properties. Molecular properties and metal analyses of the enzymes are shown in Table II. In order to assess the importance of iron, zinc, and manganese in relation to the activity of the soybean enzyme, two independently prepared samples were dialyzed against three changes of 10 mM EDTA, pH 8.0, at 4°C. Metal analyses and specific activities for the samples before dialysis are shown in Table II together with values after dialysis (in brackets). Dialysis resulted in only small decreases in specific activity (2–5%) and retention, within the error of the experiment, of the iron and zinc content of both preparations. In contrast, dialysis resulted in the complete removal of the manganese and 50% of the copper. In a second experiment, the enzyme was dialyzed against pyridine-2,6-dicarboxylate, a chelator that has been used to prepare apoenzyme forms of the red kidney bean enzyme (23) and other zinc-containing enzymes. Following dialysis against three changes of 0.1 M acetate buffer, pH 4.5, containing 0.5 M NaCl and 2 mM pyridine-2,6-dicarboxylate at 4°C, the specific activity decreased to 56% of the original and the zinc content decreased from 0.67 to 0.31 g-atom per subunit. The iron content did not change significantly (0.88 Fe/subunit before *vs.* 0.82 Fe/subunit after). Manganese and copper had been completely removed. An aliquot of this enzyme was then incubated with a large excess of $ZnSO_4$ (final concentration, 13.6 mM). After 3 h, the specific activity increased to 77% of the original and the zinc increased to 0.42 Zn/subunit (65% of the original).

TABLE II

Molecular Properties of Sweet Potato and Soybean Purple Acid Phosphatases

Property	Sweet potato	Soybean
Subunit weight[a]	55,000 ± 2,000	58,000 ± 3,000
Spectra		
λ_{max} (nm)	560	550
ϵ_{max}(M^{-1} cm^{-1})[b]	3,200 ± 300 (3,200 ± 300)	3,300 ± 300 (4,000 ± 400)

Specific activity[c,d] (U/mg)			
A	B	A	B
730–918[e]	727[f]	218 (207)[g]	208 (195)[g]

Metal content[d,h]			
A	B	A	B
Fe	1.03	0.99	0.77, 0.86 (0.83)	0.79, 0.88 (0.84)
Mn	0.58	0.66	–, 0.32 (0)	–, 0.22 (0)
Zn	0.18	0.04	0.75, 0.78 (0.63)	0.65, 0.67 (0.65)
Cu	0.11	0.08	–, 0.29 (0.14)	–, 0.12 (0.06)

Note. Metal analyses were determined by ICP-MS or ICP-AES. Standard errors were ~±3%, except for the second and the third numbers for the soybean enzyme where the error is ~±15%. These less accurate analyses were performed using external calibration with a multielemental standard as described in Methods. The number in brackets gives the metal content after dialysis against EDTA.

[a] Both enzymes are glycoproteins as evidenced by binding to concanavalin A–Sepharose. The molecular weights of the polypeptide components are 49,706 and 50,049 for the sweet potato and soybean enzymes, respectively.

[b] Calculated based on the molar concentration of protein or (in brackets) the molar concentration of iron.

[c] Specific activities determined at 25°C in 0.1 M acetate buffer, pH 4.90 (sweet potato purple acid phosphatase) and at 30°C in 0.1 M acetate buffer, pH 5.5, 0.5 M NaCl, 1 mg/ml bovine serum albumin (soybean purple acid phosphatase).

[d] Two independent preparations (A and B) of each enzyme were analysed for metal content and specific activity.

[e] Range of specific activities across the final elution profile.

[f] Specific activity was essentially constant across the final elution profile.

[g] Specific activity after dialysis.

[h] g-atom of metal per monomer of 55 kDa (sweet potato purple acid phosphatase) and 58 kDa (soybean purple acid phosphatase).

TABLE III

Substrate Specificity of Plant Purple Acid Phosphatases

Substrate	Sweet potato[a]		Soybean[b]		Red kidney bean[c]	
	k_{cat} (s^{-1}), K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat} (s^{-1}), K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat} (s^{-1}), K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
p-NPP	2160 ± 15, 95 ± 15	14	230 ± 10, 8 ± 1	29	470 ± 40, 35700 ± 5000	0.013
					77 ± 0.7, 480 ± 10	0.16
ATP	980 ± 5, 120 ± 10	5	65 ± 5, 5 ± 1	13	2700 ± 200, 430 ± 100	6.3
					1200, ≤5	≥240
ADP	310 ± 1, 180 ± 2	1.1	100 ± 5, 6 ± 1	17	530 ± 30, 7400 ± 1800	0.07
AMP	230 ± 2, 360 ± 30	0.4	74 ± 3, 9 ± 1	8.2	—	0.0002
Pyrophosphate	1070 ± 2, 75 ± 5	8.6	120 ± 5, 9 ± 1	13.3	—	0.5
β-Glycerophosphate	3370 ± 25, 490 ± 60	2.8	50 ± 2, 30 ± 4	1.7	—	0.00002
					—	0.0005
Glucose 6-phosphate	1210 ± 10, 920 ± 80	0.8	N.D.[d]	N.D.	N.D.	N.D.

[a] 0.1 M acetate buffer, pH 4.90, 25°C. k_{cat} values are calculated based on the concentration of Fe–Mn centers.
[b] 0.1 M acetate buffer, pH 5.50, containing 1 mg/ml bovine serum albumin, 25°C.
[c] For *p*-NPP, ATP, and β-glycerophosphate, constants were determined in 0.2 M succinate, 0.25 M NaCl, pH 6.50, at 30°C (top line) and in 0.1 M acetate buffer, pH 5.50, containing 1 mg/ml bovine serum albumin, 25°C (bottom line). Other constants were determined only at pH 6.50.
[d] N.D., not determined.

Steady-state kinetic constants for hydrolysis of several substrates by the purified enzymes are given in Table III. Data for the red kidney bean purple acid phosphatase are included for comparison.

Sequence analysis. The cloning strategy resulted in the cloning of two forms of sweet potato purple acid phosphatase and one form of the soybean enzyme (SP-PAP1, SP-PAP2, and SB-PAP, respectively). Comparison of the deduced N-terminal sequences of the sweet potato enzymes with that determined by direct sequencing of the purified protein shows that the isoform we have isolated corresponds to SP-PAP1 in Fig. 1. An alignment of the deduced amino acid sequences for SP-PAP1, SP-PAP2, and SB-PAP, together with that of a third sweet potato isoform reported very recently (SP-PAP3) (24) and red kidney bean purple acid phosphatase (RKB-PAP) (25), is shown in Fig. 1. While SP-PAP2, SP-PAP3, and SB-PAP exhibit high sequence identity with RKB-PAP (>74%), SP-PAP1 exhibits somewhat lower identity (66%).

DISCUSSION

The purple acid phosphatases purified in this work from sweet potato and soybean share the following characteristics with the red kidney bean enzyme: (i) a subunit weight of ~55 kDa; (ii) the presence of one g-atom of iron per subunit; (iii) purple color and λ_{max} of 550–560 nm, ϵ_{max} ~3000; (iv) the presence of 0.6–0.8 g atom of a divalent metal ion, Mn in the case of the sweet potato and Zn in the soybean; and (v) very similar N-terminal sequences. We conclude from these results that both enzymes contain binuclear metal centers similar to that in the red kidney bean. In the soybean enzyme, the center is almost certainly an FeIII–ZnII center as in the red kidney bean enzyme. This conclusion is also strongly supported by the dialysis experiments (Table II), which show that the activity correlates with the presence of Fe and Zn, but not of Mn or Cu. The sweet potato enzyme contains the first reported example of an iron–manganese binuclear center. This raises the question of the oxidation state of the centre. Analogy with the red kidney bean and mammalian purple acid phosphatases would suggest an FeIII–MnII complex. Confirmation of this awaits further analysis, for example by metal substitution. It should be noted that the Mn-containing sweet potato purple acid phosphatase isolated by Sugiura *et al.* (8) exhibited a λ_{max} of 515 nm, quite different from the present enzymes. Magnetic susceptibility measurements support the presence in our enzyme of a strongly antiferromagnetically coupled center (G. Hanson, G. Schenk, and K. Murray, unpublished results). However, the presence of smaller amounts of other binuclear centers (e.g., FeIII–ZnII and MnIII–MnII) cannot be ruled out.

Using a cloning strategy based on the conserved sequence motifs surrounding the metal ligands (2) and the known N-terminal sequences, three cDNAs were cloned and sequenced, two from sweet potato and one from soybean. As indicated under Results the three are closely related to the red kidney bean sequence. The form of sweet potato enzyme purified corresponds to one of the two cDNA isoforms rather than a mixture of the two. In summary, the result confirms the conclu-

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SP-PAP1     mrlvvVglwcLiLgLILNptkfCda GVtSSYVRKslsalpnaedVDMPwD SDVFaVPsGYNAPQQVHITQGDyeG rgVIISWtTpyDkaGanKVVYWSEN [100]
SP-PAP2      mgasrtgcyLlavvlaaVmnaaia GItSSFIRKVE------KTVDMPLD SDVFRVPPGYNAPQQVHITQGDhVG KAmIVSWVTv-DEPGSSKVVYWSEN [92]
SP-PAP3                                          DMPLD SDVFRVPPGYNvPQQVHITQGDyeG KgVIISWVTp-EEPGSktVVYWaEN [54]
SB-PAP        MGVVeGlLALALVLsacVmCNg GssSpFIRKVE------KTVDMPLD SDVFaVPPGYNAPQQVHITQGDlVG KAVIVSWVTv-DEPGSSeVhYWSEN [91]
RKB-PAP       MGVVkGlLALALVLNVvVvsNg GksSnFVRKtn------KnrDMPLD SDVFRVPPGYNAPQQVHITQGDlVG rAmIISWVTm-DEPGSSaVrYWSEk [91]

Consensus      MGVV G LALALVLNV V CN  G  SSF RKVE      KTVDMPLD SDVFRVPPGYNAPQQVHITQGD VG KAVIISWVT  DEPGSSKVVYWSEN

                                                                                          I
SP-PAP1     SksqKrAmGtVVTYkYYNYTSaFIH HCTIkdLEYdTKYYYrlGFGdakRQ FWFVTPPkpGPDVPYvFGLIGDiGQ thDSNTTLTHYEqNsaKGQaVLFmG [200]
SP-PAP2     SqhKKVArGnIrTYtYFNYTSGYIH HCTIRnLEYnTKYYYEVGiGNTTRs FWFtTPPevGPDVPYTFGLIGDLGQ SFDSNrTLTHYErNPIKGQaVLFVG [192]
SP-PAP3     SsvKrrADGvVVTYkYYNYTSGYIH HCTIkdLEYdTKYYYElGLGdakRQ FWFVTPPkpGPDVPYTFGLIGDLGQ tYDSNTTLTHYELNPVKGQslLFVG [154]
SB-PAP      SdkKKIAEGKlVTYrFFNYsSGFIH HtTIRnLEYkTKYYYEVGLGNTTRQ FWFVTPPeiGPDVPYTFGLIGDLGQ SFDSNkTLsHYELNPrKGQtVLFVG [191]
RKB-PAP     ngrKrIAkGKmsTYrFFNYsSGFIH HtTIRkLkYnTKYYYEVGLrNTTRr FsFITPPqtGlDVPYTFGLIGDLGQ SFDSNTTLsHYELsPkKGQtVLFVG [191]
                                                                                          #
Consensus   S  KKIA GKVVTY YFNYTSGFIH HCTIR LEY TKYYYEVGLGNTTRQ FWFVTPP  GPDVPYTFGLIGDLGQ SFDSNTTLTHYELNP KGQ VLFVG

            II                                III
SP-PAP1     DLSYsnRWPNHDNnRWDTWGRFsER SVAYQPWIWTAGNHEIDYAPDIGEy qPFvPFTnRYptPhEASGSgdPLWY aIKRASAHIIVLSSYSgFvKYsPQY [300]
SP-PAP2     DLSYADnYPNHDNVRWDTWGRFvER StAYQPWIWTAGNHEIDFAPEIGET kPFKPFTkRYHVPYkASGSTetFWY pIKRASAyIIVLSSYSAYGKYTPQY [292]
SP-PAP3     DLSYADRYPNHDNnRWDTWGRFvER StAYQPWIWTAGNHEIDFvPDIGET VPFKPFTHRFfmPFEsSGSTSPLWY SIKRASAHIIVMSSYSAYGtYTPQW [254]
SB-PAP      DLSYADnYPNHDNIRWDsWGRFtER SVAYQPWIWTAGNHEnhFAPEIGET VPFKPYTHRYHVPYkASqSTSPFWY SIKRASAHIIVLaSYSAYGKYTPQY [291]
RKB-PAP     DLSYADRYPNHDNVRWDTWGRFtER SVAYQPWIWTAGNHEIEFAPEInET ePFKPFsyRYHVPYEASqSTSPFWY SIKRASAHIIVLSSYSAYGrgTPQY [291]
            #  #                              #
Consensus   DLSYADRYPNHDNVRWDTWGRF ER SVAYQPWIWTAGNHEIDFAPEIGET VPFKPFTHRYHVPYEASGSTSPFWY SIKRASAHIIVLSSYSAYGKYTPQY

                          IV                                  V
SP-PAP1     KWFtsELeKVNRSETPWLIVLvHaP LYNSYeaHYMEGEaMRaiFEPYFVy YKVDIVFsGHVHsYERSERVSNVAY NIVNakCTPVsDeSAPVYITIGDGG [400]
SP-PAP2     KWLEeELPKVNRtETPWLIVLMHSP wYNSYNyHYMEGETMRVMYEPWFVQ hKVDlVFAGHVHAYERSERVSNVAY dIVNGkCTPVrDQSAPVYITIGDGG [392]
SP-PAP3     KWLqgELPKVNRSETPWLIVLMHcP MYsSYvhHYMEGETMRVLYEPWFVe YKVDVVFAGHVHsYERtERVSNVAY NIVNGLCsPkNDsSAPVYITIGDGG [354]
SB-PAP      KWLEKELPKVNRtETPWLIVLMHSP wYNSYNyHYMEGETMRVMYEPWFVQ YKVDVVFAGHVHAYERSERVSNVAY NIVNGLCaPVNDkSAPVYITIGDGG [391]
RKB-PAP     tWLkKELrKVkRSETPWLIVLMHSP LYNSYNhHFMEGEaMRtkFEaWFVk YKVDVVFAGHVHAYERSERVSNIAY kItNGLCTPVkDQSAPVYITIGDaG [391]
                          #                                   # #
Consensus   KWLEKELPKVNRSETPWLIVLMHSP LYNSYN HYMEGETMRVMYEPWFVQ YKVDVVFAGHVHAYERSERVSNVAY NIVNGLCTPVNDQSAPVYITIGDGG


SP-PAP1     NsEGLAseMTQPQPsYSAFREASFG HGIFDIKNRTHAHFSWHRNQDGasV EADSLWLLNRYWaseDaSsmSAM  [474]
SP-PAP2     NlEGLATNMTdPQPeYSAFREASFG HatLDIKNRTHAyYSWHRNQDGyAV EADSMWvsNRFWHPVDDSTttkL  [466]
SP-PAP3     NsEGLATeMTQPQPsYSAYREASFG HGIFDIKNRTHAHFgWHRNQDGlAV EgDSLWFiNRYWmskEEasvSAv  [428]
SB-PAP      tlEGLATNMTePQPkYSAFREASFG HaIFDItNRTHAHYSWHRNQDGVAV EADSLWsFNRYWHPVDDSTahvsh [466]
RKB-PAP     NygvidsNMiQPQPeYSAFREASFG HGmFDIKNRTHAHFSWnRNQDGVAV EADSvWFFNRhWyPVDDST      [461]

Consensus   N EGLATNMTQPQP YSAFREASFG HGIFDIKNRTHAHFSWHRNQDGVAV EADSLWFFNRYWHPVDDST SA
```

FIG. 1. Comparison of the deduced amino acid sequences of three sweet potato purple acid phosphatase isoforms: SP-PAP1 and SP-PAP2 (determined in the present work) and SP-PAP3 (determined by Durmus *et al.* (24)), soybean purple acid phosphatase (SB-PAP), and red kidney bean purple acid phosphatase (RKB-PAP) (25). Residues matching the consensus shown below the aligned sequences are denoted with upper-case characters; nonmatching amino acids are written in lower case. Numbers in brackets at the end of each line indicate the residue numbers. The five conserved regions (in bold) contain the seven invariant amino acids (#) involved in coordination of the metals in the binuclear center. Signal peptides are shown in italics.

sion based on metal analysis that the sweet potato and soybean enzymes are members of the binuclear purple acid phosphatase family.

A number of possible explanations can be offered for the discrepancies between the present result and the literature for the metal content of sweet potato and soybean purple acid phosphatases: (i) partial loss of divalent metal ion during purification (even for the well-characterized red kidney bean enzyme, which is recognized to contain 2 g-atoms of iron and 2 g-atoms of zinc per dimer, a shortfall of metals is usually found (3); (ii) exchange of one divalent metal for another, with retention of activity (the fact that the zinc ion of the red kidney bean enzyme may be readily exchanged for other divalent metals with retention of activity (23) is well established); (iii) the possibility of different metal contents depending on cultivar, soil composition, culture method, and purification procedure (in the present study, the soybean purification involved chromatography on Con A–Sepharose, a procedure which could lead to uptake of manganese; however, the dialysis experiments showed that all of the manganese could be removed from the soybean preparation with minimal loss of activity, and Con A–Sepharose was avoided in purifying the sweet potato enzyme); and (iv) different forms of the enzyme may show different specificity in the binding of metal ions. Since the completion of our work, Durmus *et al.* (24) reported the sequences of two sweet potato purple acid phosphatase cDNAs, one of which (designated "spPAP_{iso}") corresponds to our SP-PAP2 and a second (designated "sp-PAP") which is different from either of our isoforms. The enzyme corresponding to this latter form was purified by Durmus *et al.* and shown to contain an FeIII–ZnII center very similar to that in the red kidney bean enzyme. These results, together with ours, demonstrate conclusively that different isoforms of the sweet potato purple acid phosphatase exist, and that at least

two of the isoforms contain different metal centers. Whether the enzyme corresponding to the third isoform (SP-PAP2/spPAP$_{iso}$) is the enzyme characterized by the Japanese group (6–8) and whether it possesses yet another type of metal center, for example, Mn–Mn, remains to be established.

As shown in Table III, both sweet potato and soybean enzymes utilize a range of phosphate ester and anhydride substrates. They hydrolyze activated substrates like ATP and *p*-NPP and unactivated substrates like β-glycerophosphate with comparable efficiencies. In this respect, the enzymes resemble lysosomal and prostatic acid phosphatases. In contrast, the red kidney bean enzyme shows a marked preference for activated substrates at both pH 5.5 and 6.5. The result is surprising, especially in view of the similarities between the red kidney bean and soybean enzymes in terms of their molecular properties. Mammalian purple acid phosphatases also exhibit a preference for activated phosphate esters and anhydrides over stable esters (26). However, the overriding preference of the red kidney bean enzyme for ATP is unique among the purple acid phosphatases studied so far. Determination of the three-dimensional structure of either the sweet potato or the soybean enzyme will facilitate the identification of differences among the active sites which may account for the observed specificity differences and aid in the identification of physiologically relevant substrates.

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