

Soybean Root Nodule Acid Phosphatase¹

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Acid phosphatases are ubiquitous enzymes that exhibit activity against a variety of substrates *in vitro*, although little is known about their intracellular function. In this study we report the isolation, characterization, and partial sequence of the major acid phosphatase from soybean (*Glycine max* L.) root nodules. The phosphatase was purified predominantly as a heterodimer with subunits of 28 and 31 kD; homodimers of both subunits were also observed and exhibited phosphatase activity. In addition to the general phosphatase substrate, *p*-nitrophenyl phosphate, the heterodimeric form of the enzyme readily hydrolyzed 5'-nucleotides, flavin mononucleotide, and O-phospho-L-Tyr. Low or negligible activity was observed with ATP or polyphosphate. Purified nodule acid phosphatase was stimulated by magnesium, inhibited by calcium and EDTA, and competitively inhibited by cGMP and cAMP with apparent K_i values of 7 and 12 μ M, respectively. Partial N-terminal and internal sequencing of the nodule acid phosphatase revealed homology to the soybean vegetative storage proteins. There was a 17-fold increase in enzyme activity and a noticeable increase in protein levels detected by immunoblotting methods during nodule development. Both of these parameters were low in young nodules and reached a peak in mature, functional nodules, suggesting that this enzyme is important for efficient nodule metabolism.

General phosphohydrolases are classified as alkaline phosphatases or acids based solely upon whether their optimal activity is above or below pH 7.0 (Vincent et al., 1992). Whereas plant alkaline phosphatases have been characterized as having specific metabolic roles, most plant ACPs have been shown to hydrolyze a wide variety of phosphorylated substrates *in vitro*, making their cellular role(s) difficult to define (for review, see Duff et al. [1994]). One method of determining the substrate specificity of an ACP is to determine the kinetic parameters of specificity such as V_{max}/K_m with numerous substrates. This approach was taken by Duff et al. (1989a, 1989b, 1991) to characterize an ACP from *Brassica nigra* suspension-cultured cells as a PEP phosphatase.

The difficulty in classifying plant ACPs is further compounded by a lack of available sequence information. Only

three plant ACP genes have been cloned and sequenced. The first plant gene to be classified as an ACP was from tomato, although interest in it was primarily due to its tight genetic linkage to a root-knot nematode-resistance gene rather than to its enzymic activity (Aarts et al., 1991; Erion et al., 1991; Williamson and Colwell, 1991). The other plant AP that has been cloned and sequenced is the soybean VSP (Staswick 1988; Dewald et al., 1992), which has been shown to possess some phosphatase activity (Dewald et al., 1992).

Soybean (*Glycine max* L.) root nodules export nitrogen in the form of the ureides allantoin and allantoic acid, which are derived from de novo-synthesized purines (Schubert, 1981, 1986; Atkins et al., 1982; Christensen and Jochimsen, 1983; Doremus and Blevins, 1988). What makes this pathway particularly interesting in terms of plant phosphatases is that the first step in the conversion of purines (namely 5' \times IMP) to ureides is the removal of the 5'-phosphate group. Consistent with the difficulty in classifying ACPs, one model suggests that the enzyme responsible for phosphate removal is a nonspecific ACP (EC 3.1.3.1; Guranowski, 1982), whereas another suggests that 5'-nucleotidase (EC 3.1.3.5) performs this role (Christensen and Jochimsen, 1983). A 5'-nucleotidase activity has been examined in soybean root nodule crude extracts (Doremus and Blevins, 1988) and partially purified (Ostergaard et al., 1991). The level of 5'-nucleotidase activity in soybean root nodule extracts is dramatically higher than in two other ureide exporters, *Vigna unguiculata* and *Phaseolus vulgaris*, and was estimated to be 50 times greater than the activity of phosphoribosylpyrophosphate aminotransferase, which is presumed to be the rate-limiting enzyme in ureide production (Doremus and Blevins, 1988). One explanation for this discrepancy is that the enzyme responsible for 5'-nucleotidase activity in soybean root nodules is also active on other substrate(s) *in vivo*. To better understand the role of ACPs in plant metabolism, we have purified, characterized, and partially sequenced the protein. The ontogeny of enzyme activity and expression have also been determined for this major ACP activity in soybean root nodules.

MATERIALS AND METHODS

Soybean (*Glycine max* L. Merr. cv Hobbit) seeds were inoculated with *Bradyrhizobium japonicum* strain USDA 110. Three or four seeds were planted in 18-cm sand-filled pots and grown in a greenhouse (Sarath et al., 1986). Nodules

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Abbreviations: ACP, acid phosphatase; CM, carboxymethyl; col-vol, column-volume; ConA, concanavalin A; pNPP, paranitrophenyl phosphate; TFA, trifluoroacetic acid; VSP, vegetative storage protein.

were harvested from 30- to 35-d-old plants, thoroughly washed, and stored at -20°C .

Purification of the Nodule ACP

Twenty-five grams of nodules was ground with a mortar and pestle in 80 mL of 0.1 M Tris-HCl, pH 7.0, containing 2.5 g of PVP, 1 mM MgCl_2 , 1 mM CaCl_2 , 1 mM MnCl_2 , 1 mM β -mercaptoethanol, 5 μM L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]-agmatine (E-64), and 1 mM PMSF. The homogenate was passed through two layers of Miracloth (Calbiochem) and centrifuged for 30 min at 15,000g, and the supernatant (crude extract) was reserved.

A 2.5- \times 5-cm (25-mL) column of ConA-Sepharose was equilibrated in buffer A (0.1 M Tris-HCl, pH 7.0, containing 1 mM MgCl_2 , 1 mM CaCl_2 , 1 mM MnCl_2 , and 0.5 M NaCl). Crude extract was applied to the column in 1-col-vol aliquots and allowed to bind for 15 min. The column was then washed with 6 col-vols of buffer A. Bound protein was eluted with 3 col-vols of 0.1 M Tris-HCl, pH 7.0, containing 1 mM MgCl_2 , 0.5 M NaCl, and 0.2 M methylmannopyranoside.

The eluted sample from ConA-Sepharose chromatography was concentrated in a stirred pressure cell over a membrane (YM-10, Amicon Beverly, MA), and dialyzed into 30 mM NaAc, pH 5.5. The dialyzed sample was passed through a CM-MemSep cartridge (Waters; 1 col-vol = 1 mL) using a BioCad workstation (PerSeptive Biosystems, Framington, MA). The cartridge was equilibrated in 30 mM NaAc, pH 5.5, and developed with a linear gradient from 0 to 0.5 M NaCl in 30 mM NaAc, pH 5.5, over 40 col-vols at 3 mL min^{-1} .

Active fractions from chromatography were pooled and loaded on a MonoS 5/5 column (Pharmacia; 1 col-vol = 1 mL) equilibrated in 30 mM NaAc, pH 5.0. The three dimeric forms of ACP were resolved with a linear gradient from 0 to 0.8 M NaCl in 30 mM NaAc, pH 5.0, over 50 col-vols at 1 mL min^{-1} . Fractions containing the ACP heterodimer were pooled for activity determinations.

Fractions containing ACP $\alpha\alpha$ - or $\beta\beta$ -homodimers were separately pooled, lyophilized, dissolved in reverse-phase buffer A (2% MeCN in 0.1% TFA), and chromatographed on a 4.6- \times 100-mm reverse-phase column (1 col-vol = 1.662 mL Poros R2H, PerSeptive Biosystems). ACP was eluted with a linear gradient from 0 to 70% buffer B (90% MeCN containing 0.09% TFA) over 30 col-vols at 7.5 mL min^{-1} .

Enzymatic Digestion of ACP Subunits

Approximately 30 μg of ACP $\alpha\alpha$ - or $\beta\beta$ -homodimers purified by reverse-phase HPLC were digested with 3 μg of trypsin for 16 h at 37°C . Tryptic fragments were separated on a 2.1- \times 250-mm C18 column (model 218TP52, VYDAC, Hesperia, CA; 1 col-vol = 0.866 mL) with a linear gradient from 2% MeCN in 0.1% TFA to 60% buffer B (90% MeCN in 0.09% TFA) over 15 col-vols at 0.2 mL min^{-1} on a microbore HPLC (Isco, Lincoln, NE). A_{214} was monitored and peaks were collected manually.

Protein Sequencing

Purified ACP was fractionated by 10% SDS-PAGE and blotted onto membranes (Immobilon P, Millipore; Wilson and Yuang, 1988). Membranes were stained with Amido black, and bands corresponding to ACP α and β subunits were excised and sequenced (Procise 494 sequencer, Applied Biosystems) at the University of Lincoln Protein Core Facility using manufacturer-supplied protocols. Peptide fragments obtained by reverse-phase chromatography were directly applied to polybrene-coated micro-TFA sequencing discs (Applied Biosystems) and sequenced.

Enzyme Activity Assays, SDS-PAGE, and Western Analysis

Protein concentration was determined by the BCA assay (Pierce) employing BSA as a standard.

Phosphatase activity against pNPP was determined by monitoring the release of *p*-nitrophenol at 405 nm. The release of phosphate from other substrates was monitored by the method of Fiske and Subbarow (1925) using a kit from Sigma. Activity determinations were routinely performed in triplicate at 25 or 30°C in 0.05 M Mes-NaOH, pH 6.0, containing 1 mM MgCl_2 .

Cold SDS-PAGE in 10% gels was performed according to the method of Laemmli (1970), incorporating the modifications of Hummel et al. (1996), except that the substrate was omitted from the separating gel. All steps, including treatment with SDS loading buffer, were performed at 4°C . After electrophoresis, gels were rinsed in H_2O for 10 min, followed by 30 min in 2.5% (v/v) Triton X-100 in 0.05 M Mes-NaOH, pH 6.0, containing 1 mM MgCl_2 . Gels were subsequently stained for activity with 4-methylumbelliferyl phosphate (5 mg 50 mL $^{-1}$) in 0.05 M Mes, pH 6.0, containing 1 mM MgCl_2 . Activity bands were excised, macerated, boiled in SDS loading buffer, and re-electrophoresed in standard 10% Laemmli gels. Gels were then stained for total protein with Coomassie blue and/or silver.

Soybean leaf extract from depodded plants (2.5 μg of protein), soybean root nodule crude extract (100 μg of protein), and 100 ng of purified nodule ACP heterodimer were run on a 12% SDS-PAGE gel and blotted to a nitrocellulose membrane. The membrane was probed with a polyclonal antiserum raised against deglycosylated soybean VSP α (Staswick, 1988).

Time Course of Appearance of ACP Activity and Proteins during Nodule Ontogeny

Nodulated plants were raised from seed as described above and nodules were harvested at 3-d intervals starting 9 d after planting. At the earliest stages nodules were white, but became deep-red by d 21 after planting. Nodules were also harvested from older plants, at a stage when the innermost central region of the nodules had begun to turn green (d 52). Harvested nodules were extracted by grinding in a mortar and pestle in 50 mM Mes-NaOH, 5 mM MgCl_2 , 1 mM PMSF, pH 6.0, and centrifuged to pellet cellular debris. The clarified supernatant was used as the source of enzyme and protein. Protein concentrations and enzymatic analyses using 5'-GMP as a substrate were de-

terminated as described above. Nodule ACP subunits were determined by immunoblotting of SDS-PAGE-separated nodule extracts probed with a polyclonal antiserum raised against deglycosylated soybean VSP α (Staswick, 1988).

RESULTS

Purification of Nodule ACP

Nodule ACP was purified 300-fold to near homogeneity by ConA-Sepharose chromatography followed by HPLC cation exchange with CM-MemSep and MonoS columns (Table I; Fig. 1). The enzyme bound to ConA in the presence of 0.5 M NaCl and eluted with 0.5 M NaCl plus 0.2 M methylmannopyranoside. The ConA step effectively served to remove 90% of the contaminating proteins, including leghemoglobins. Nodule ACP was the major protein that bound to CM-MemSep at pH 5.5. MonoS chromatography primarily served to resolve the CM-MemSep-binding phosphatase activity into three fractions (Fig. 1). Estimates of chromatographic peak areas indicate that fractions I and III each comprised approximately 25% of the total ACP, and fraction II comprised approximately 50%. The final purified ACP fraction II was used for all subsequent kinetic determinations.

SDS-PAGE of fraction II revealed the presence of 28- and 31-kD polypeptides of equal staining intensity (Fig. 2A), suggesting that under the conditions employed nodule ACP exists as an even-numbered multimer of 28- and 31-kD subunits. Fraction I contained only the 31-kD band, whereas fraction III contained only the 28-kD band (not shown). To confirm the relationship of the ACP subunits to the observed chromatographic activity profiles, cold SDS-PAGE (Hummel et al., 1996) was employed. Under these conditions some proteins maintain their subunit structure and activity, and are therefore a useful means for determining the relationships of observed enzyme isoforms. Samples from CM-MemSep chromatography, which contained all three fractions of nodule ACP, were subjected to cold SDS-PAGE. The three fractions of ACP were resolved under these conditions and retained their activity (Fig. 2B). The activity bands migrated to a position corresponding to the migration of molecular mass standards of approximately 70 kD. When these three activity bands were individually excised, denatured by heating in the presence of SDS, and re-electrophoresed, they displayed the presence of polypeptides of 31, 31 and 28, and 28 kD, respectively (Fig. 2C). The data presented in Figure 2, A and B, suggest that ACP exists as dimers of the 31- and 28-kD subunits.

Table I. Purification of nodule ACP

Step	Total Protein	Total Activity	Specific Activity	Purification	Recovery
	mg	units	units mg ⁻¹	-fold	%
Crude	490	73.2	0.15	1	100
ConA	56	26.4	0.47	3.1	36
CM-Memsep	1.2	28.5	23.9	159	39
MonoS	0.3	13.5	45.0	300	18

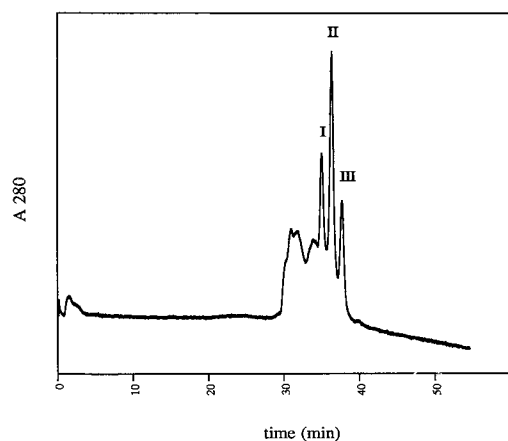


Figure 1. MonoS chromatography of nodule ACP. Fractions exhibiting ACP activity after CM-MemSep chromatography were pooled and loaded on a MonoS 5/5 column. The column was developed with a linear gradient from 0.0 to 0.5 M NaCl in 30 mM NaAc, pH 5.0, over 50 col-vols. Column effluent was monitored at 280 nm and assayed for ACP activity using pNPP. Fractions containing ACP activity were collected, pooled, and labeled as fractions I, II, and III.

This was confirmed by the data presented in Figure 2C, which indicated that the three observed forms of nodule ACP are the three possible dimeric combinations of the 28-kD α -subunits and the 31-kD β -subunits.

Polyclonal antibodies to deglycosylated VSP α were used as a probe for western analysis of nodule ACP (Fig. 2D), and both subunits were detected with the antibody. The α - and β -subunits of nodule ACP were the only cross-reacting bands in the nodule crude extract; bands corresponding to the soybean leaf VSP subunits were not detected in 100 μ g of soluble nodule protein.

pH Optimum Determination

Activity of nodule ACP $\alpha\beta$ against pNPP was assayed in 0.1 M Mes-NaOH from pH 5 to 7. The enzyme was active over this entire pH range and exhibited a rather broad pH optimum, with greater than 80% maximal activity from pH 5.8 to 6.4.

Substrate Specificity of Nodule ACP

Because the physiologically relevant substrate(s) for nodule ACP was not known, the enzyme was assayed for activity against a variety of mono-, di-, and polyphosphorylated substrates. The highest activity was observed with the monophosphorylated substrates 5'-GMP, 5'-AMP, and FMN (Table II). Several other compounds were also dephosphorylated by the purified nodule ACP, albeit at a much lower rate compared with 5'-GMP. The enzyme dephosphorylated ADP, PPI, and O-phospho-L-Tyr at similar rates under the conditions of the assay. Low or negligible activity was observed with polyphosphorylated substrates such as ATP and tetrapolyphosphate. Other monophosphorylated compounds, namely cAMP, PEP, naphthol-AS-TR-phosphate, O-phospho-L-Ser, and O-phospho-L-Thr, were apparently poor substrates (Table II).

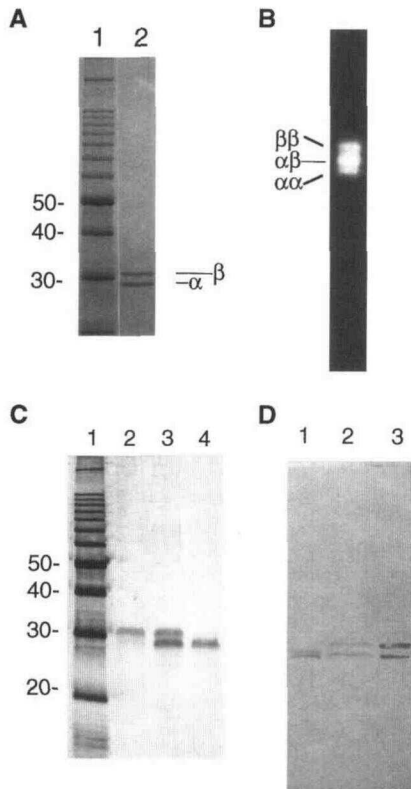


Figure 2. SDS-PAGE and immunodetection of nodule ACP. A, SDS-PAGE of purified nodule ACP heterodimer. Lane 1, 10-kD ladder; lane 2, purified nodule ACP, 10 μ g of protein from fraction II shown in Figure 1. B, CM-MemSep-purified ACP (40 μ g of protein) was subjected to cold SDS-PAGE and stained for activity using 4-methylumbelliferyl phosphate. Three zones of activity exhibiting fluorescence were detected by UV-transillumination and are labeled as $\beta\beta$, $\alpha\beta$, and $\alpha\alpha$, respectively. C, The three regions displaying ACP activity from the gel shown in Figure 2B were separately excised, denatured, and reelectrophoresed in 12% gels. Gels were stained with silver to detect proteins. Lane 1, 10-kD ladder; lane 2, $\beta\beta$; lane 3, $\alpha\beta$; and lane 4, $\alpha\alpha$. D, Western blot of leaf and nodule proteins probed with anti-VSP antibodies. Lane 1, Depodded soybean leaf crude extract (2.5 μ g); lane 2, root nodule crude extract (100 μ g); and lane 3, purified ACP heterodimer (100 ng) were separated by SDS-PAGE and electroblotted to a nitrocellulose membrane. Blotted proteins were probed with polyclonal antibodies raised in rabbits to soybean VSP α .

Based on these data, kinetic parameters for the three substrates most actively dephosphorylated were determined and are shown in Table III. The highest affinity was observed for 5'-AMP ($K_m = 0.08$ mM). The observed K_m values for 5'-GMP and FMN were somewhat higher, 0.15 and 0.36 mM, respectively. The V_{max} values for 5'-GMP were about twice those for 5'-AMP and nearly 10-fold greater than that calculated for FMN. Corresponding V_{max}/K_m values for 5'-AMP and 5'-GMP were 1700 and 1540, respectively, compared with 69 for FMN.

Preliminary data indicated that cyclic nucleotides at micromolar levels substantially inhibited hydrolysis of pNPP by the purified nodule ACP. To determine the mode of inhibition, the method of double-reciprocal plots was used.

Table II. Activity of nodule ACP against a variety of phosphorylated substrates

Activities were determined in triplicate using 0.5 μ g of enzyme/assay and are expressed as a percentage relative to the release of phosphate from 5'-GMP.

Substrate	Relative Activity
	% of 5'-GMP
5'-GMP	100
5' AMP	59
FMN	27
ADP	10
PPi	15
O-Phospho-L-tyrosine	15
ATP	3
Tetrapolyphosphate	4
cAMP	<2
PEP	4
O-Phospho-L-serine	<2
O-Phospho-L-threonine	<2

Double-reciprocal plots of nodule ACP activity using pNPP as the substrate in the presence or absence of cAMP and cGMP are shown in Figure 3. As indicated from these plots, nodule ACP is competitively inhibited by these cyclic nucleotides. Apparent K_i values were found to be 7 μ M for cGMP and 12 μ M for cAMP.

Effects of Metals and Chelating Agents on Nodule ACP Activity

Nodule ACP was assayed for activity against pNPP in the presence or absence of various metals and chelating agents. The most notable effects were the stimulation of activity by magnesium and its inhibition by calcium. The addition of 1 mM $MgCl_2$ stimulated activity to 152% of control, whereas 1 mM $CaCl_2$ reduced activity to 55% of control; 1 mM $ZnCl_2$ and $MnCl_2$ had little or no effect on activity; 1 mM $NaVO_3$, NaW , EDTA, and 1,10-phenanthroline were all inhibitory, reducing activity to 30, 31, 29, and 66% of the control, respectively.

Sequence Homology between Nodule ACP and Other Plant Phosphatases

The N-terminal 20 amino acids of both the 28-kD α -subunits and the 31-kD β -subunits were found to be identical and exhibited the sequence IPEVS CQSWR

Table III. Kinetic properties of nodule ACP

Kinetic constants were determined in triplicate using 0.5 μ g of purified enzyme/assay.

Substrate	K_m	V_{max}	V_{max}/K_m
	mM	units $mg^{-1}a$	
5'-AMP	0.08	136	1700
5'-GMP	0.15	231	1540
FMN	0.36	25	69
pNPP	13.25	159	12

^a One unit = 1 μ mol of phosphate released per minute at 25°C in 0.05 M Mes-NaOH, pH 6.0, plus 1 mM $MgCl_2$.

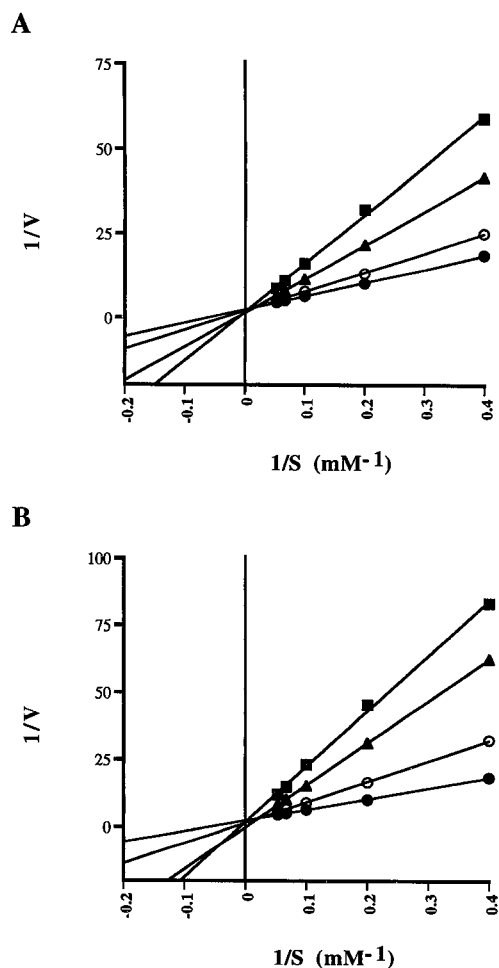


Figure 3. Double-reciprocal plots to determine the mode of inhibition by cAMP and cGMP. Activity against pNPP was determined using a continuous spectrophotometric assay in the presence of 50, 25, 5, and 0 μM cAMP (A) or cGMP (B). The common intercept of the lines at the $1/V$ axis is indicative of competitive inhibition. Velocities are given in arbitrary units. \bullet , No inhibitor; \blacksquare , 50 μM cGMP; \blacktriangle , 25 μM cGMP; and \circ , 5 μM cGMP.

LGVEA HNVID. Tryptic digestion of the individual subunits followed by reverse-phase HPLC of the digests did not reveal differences in the resulting peptide profiles (not shown). However, peptides were collected and some were analyzed by amino acid sequencing. Of the peptides analyzed, one (Tryptic 13) provided a 19-residue sequence. A database search of the 20 N-terminal amino acids of nodule ACP and a 19-residue tryptic fragment revealed homology to the soybean VSPs, a tomato ACP, and a cyclic-nucleotide binding phosphatase from potato tubers (Table IV). The highest homology was observed with soybean VSP α , in which 27 of the 39 (69%) residues were identical.

Time Course of Appearance of Nodule ACP Activity and Proteins

ACP activity using 5'-GMP as a substrate was determined in extracts obtained from nodules of increasing age. At the earliest time point (d 9), the nodules were white, but

visible on the crown of the roots. Nodules became progressively larger and deeper red. At the last time point (d 52) root nodules had apparently entered the senescent phase, as observed by greening of the innermost regions of the harvested nodules.

ACP activity increased about 17-fold during nodule development (Fig. 4A) from d 9 through 27 after planting, and decreased in old nodule extracts. This significant enhancement in activity was observed with 5'-GMP as the substrate. Extracts from nodules obtained from very young plants displayed a specific activity of 15.5 $\text{nmol Pi min}^{-1} \text{mg}^{-1}$ protein released to 260 $\text{nmol Pi min}^{-1} \text{mg}^{-1}$ protein for nodules obtained from 27-d-old plants. There was almost a 30% decline in the ACP specific activity in extracts from older nodules (Fig. 4A).

This change in enzyme activity was mirrored in the appearance of ACP proteins as detected by immunoblotting (Fig. 4B). At d 9 and 12, there was a faint amount of protein cross-reacting at the position of the 28-kD subunit, but the 31-kD subunit was not readily detected. By d 15 both subunits were detected and the amounts appeared to increase with nodule age to d 27. There appeared to be a decrease in the amount of protein cross-reacting to the antibodies in extracts from nodules obtained from 53-d-old plants. Therefore, both the enzyme activity and the immunoblotting data indicate that this enzyme is enhanced during soybean root nodule ontogeny.

DISCUSSION

The major soybean root nodule ACP was purified and shown to possess high specificity for the dephosphorylation 5'-nucleotides. Analysis of the time course of the appearance of 5'-nucleotidase activity and protein suggests that this enzyme is developmentally regulated and is enriched in active nitrogen-fixing root nodules. Nodule ACP was purified as a dimer and both subunits possessed an identical amino acid sequence for their first 20 residues. A comparison of these partial amino acid sequence data revealed homology to the soybean VSPs.

Table IV. Protein sequence homology between nodule ACP and other plant phosphatases

Sequence alignments were performed using a BLAST server available through the ExPASy-Swiss-Prot database. Amino acid residues identical to those of the nodule ACP are indicated as a dot (·).

Protein	Residue No.	Sequence
Nodule ACP	1-20	IPVEVCQSWRLGVEAHNVID
Soybean VSP α	34-53	T· · · K·A· · · · · A· · · · · IFG
Soybean VSP β	38-57	SS· · K·A·F· · · · · A· · · · · IRA
Tomato phosphatase	38-57	RD·L·K·TT· · · FV· · TN·L·SP
Potato phosphatase	1-20	V·QLD·L· · · · · A· · TN·L·QN
Nodule ACP	T13 ^a	IIGNIGDQWSDLLGTNTGD
Soybean VSP α	219-237	·V·I· · · · · · · · · · · GHR·E
Soybean VSP β	222-240	·V·I· · · · · · · · · · · DHR·E
Tomato phosphatase	224-242	·V· · S· · · · · · · · · · · SSMSY

^a Tryptic peptide 13, Generated from the 31-kD subunit.

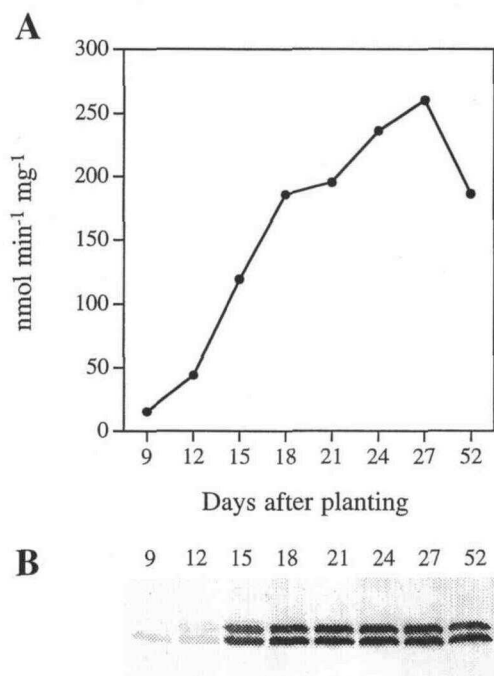


Figure 4. Time course of appearance of ACP activity and protein subunits during nodule ontogeny. A, Enzyme activity in nodule extracts was assayed using 5'-GMP as a substrate. B, Immunodetection of ACP proteins in extracts from each harvest date. Equal amounts of nodule proteins (100 $\mu\text{g lane}^{-1}$) from each harvest date were separated by SDS-PAGE in 12% gels. The amounts of ACP proteins were subsequently determined by immunoblotting.

It appears that nodule ACP was previously designated as a nodule variant of the VSPs, based on its similar size and cross-reactivity with anti-VSP antibodies on western analysis (Staswick, 1988; Staswick et al., 1994). Data shown in Figure 2D and those obtained from amino acid sequencing of the two nodule ACP subunits indicate that neither of the VSP subunits were detected in the nodule extracts that were analyzed in this study. It is possible that VSPs are expressed at low levels in root nodules and that the ACP polypeptides predominate in nodule tissue extracts. These data suggest that these two proteins have different metabolic roles within the various organs of the soybean plant. Support for this hypothesis comes from the enzymic properties of the nodule ACP. These data clearly indicate that nodule ACP is not simply a VSP variant. DeWald et al. (1992) showed that soybean VSPs functioned, at least in vitro, as phosphatases. In marked contrast to the nodule ACP, soybean VSPs were most active against polyphosphates such as ATP and tetrapolyphosphate. Additionally, the highest activity reported for the various VSP dimers ranged from 0.3 to 10 units mg^{-1} , compared with 231 units mg^{-1} for nodule ACP with 5'-GMP.

Staswick et al. (1994) purified a novel ACP from soybean leaves that was increased (as are VSPs) upon pod removal. This enzyme had a specific activity of 1350 units mg^{-1} when pNPP was used as a substrate and comprised approximately 0.2% of the total leaf protein in depodded plants. Although VSPs may reach levels as high as 40% of

the total leaf protein in depodded plants, they represent only about 0.1% of the total activity using pNPP as substrate (Staswick et al., 1994). Thus, if they function as phosphatases in vivo, the contribution of VSPs to the total leaf phosphatase activity is slight. It seems likely, as has been suggested by Staswick et al. (1994), that VSPs function primarily as storage proteins.

To explain the high homology of soybean VSPs to the tomato AP, Staswick et al. (1994) suggested that VSPs might be derived from a phosphatase gene, possibly exploiting an intact system of temporal and/or spatial regulation, and the slight phosphatase activity of the VSPs may simply be vestigial.

The nodule ACP also displayed sequence homology to a cyclic nucleotide-binding phosphatase from potato tubers (Polya and Wettenhall, 1992). Like nodule ACP, the potato phosphatase is an approximately 60-kD dimeric, glycosylated enzyme with the highest specificity for 5'-nucleoside monophosphates. Polya (1975) demonstrated that the potato phosphatase, as well as similar enzymes from wheat seedling leaves (Polya, 1974) and silver beet leaves (Polya and Hunziker, 1987), were competitively inhibited by cyclic nucleotides with low micromolar K_i values. Similarly, both cAMP and cGMP were shown to be competitive inhibitors of nodule ACP, with apparent K_i values of 7 and 12 μM for cGMP and cAMP, respectively.

Based on its chromatographic characteristics, stimulation by magnesium and inhibition by calcium, pH optimum, and substrate specificity, the enzyme we have designated as nodule ACP appears to be the same activity previously described as a 5'-nucleotidase, which was partially purified and characterized by Ostergaard et al. (1991). The authors reported resolving the activity into four isoforms, each with an apparent native molecular mass of 70 kD; however, no indication was given as to a dimeric nature of the enzyme, and no SDS-PAGE data was provided, making it difficult to confirm the relationship of nodule ACP to the various 5'-nucleotidase isoforms.

ACP activity has also been reported in the peribacteroid space of soybean nodules (Bassarab and Werner, 1989). This enzyme displayed a pH optimum of 5.5 and substantial activity in the absence of magnesium. Specific activities for the hydrolysis of ATP, GTP, PPI, and PEP were quite similar, and ranged between 132 and 189 units mg^{-1} protein (Bassarab and Werner, 1989). These values are significantly different from those observed for the purified nodule ACP, which exhibited poor activities when ATP and PEP were used as substrates. The relationships between these reported nodule APs are not readily apparent, and need to be investigated further.

In an attempt to understand the role of this enzyme in root nodule metabolism, we studied the ontogeny of the appearance of protein and enzymic activities in soybean plants nodulated by an effective strain of *B. japonicum* USDA-110. There was a 17-fold increase in enzyme activity over the time frame when nodule metabolism adapts to nitrogen fixation and transport for fixed nitrogen. Similar increases have been documented for other enzymes involved in the de novo synthesis of purines (Schubert, 1981,

1986; Reynolds et al., 1982). More recently, Atkins et al. (1997) demonstrated that both the plastids and mitochondria have all of the enzymes required for the de novo synthesis of purines; however, the enzymes converting XMP to xanthine appeared to be in the soluble fraction. Datta et al. (1991) showed that the principal location of xanthine dehydrogenase, which oxidizes xanthine to uric acid, is the uninfected cells. This would imply that xanthine is probably generated in the infected cells by the sequential action of a 5'-nucleotidase, which dephosphorylates XMP to xanthosine, and a 5'-nucleosidase, which converts xanthosine to xanthine. Atkins et al., (1989) have purified a soluble 5'-nucleosidase from cowpea root nodules that appears to preferentially deribosylate 5'-nucleosides. The observed characteristics of the nodule ACP reported in this study would strongly suggest that this enzyme is the 5'-nucleotidase involved in the generation of 5'-nucleosides from 5'-nucleotides during ureide biosynthesis in soybean root nodules.

In addition to its postulated role in ureide biosynthesis, nodule ACP could conceivably participate in the metabolism of flavin nucleotides. Soybean root nodules, particularly in the symbiosome space, have been shown to be enriched in riboflavin and FMN; however, the source and metabolism of these flavins are poorly understood (Fehling et al., 1992; Werner, 1992; Mellor and Collinge, 1995). Fehling et al. (1992) have shown that the level of riboflavin is positively correlated to nodule effectiveness. The relatively high affinity of nodule ACP for FMN presents the possibility that one of its *in vivo* roles is the dephosphorylation of FMN to riboflavin. Elaboration of the site(s) of localization and regulation of this phosphatase will aid in defining its role in nodule metabolism.

We are currently employing a PCR-based strategy with primers designed from the nodule ACP protein sequence to isolate the cDNA and gene for nodule ACP. It is anticipated that the gene and cDNA sequence of nodule ACP will provide a means to understand its expression and regulation. It will be particularly interesting to determine if the expression of nodule ACP is regulated by similar mechanisms as those shown for VSPs (Staswick, 1994).

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