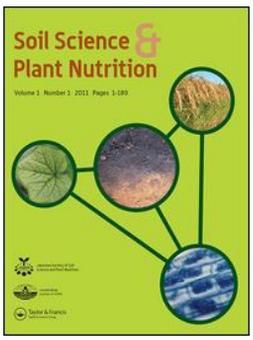

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Properties of secretory acid phosphatase from lupin roots under phosphorus-deficient conditions

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Key words: lupin (*Lupinus albus* L. cv. Kievskij), phosphorus-deficient, secretory acid phosphatase (S-APase)

Abstract

Phosphorus deficiency induces the synthesis of acid phosphatases in roots of lupin and other plant species. In this study we examined the induction of secretory acid phosphatase (S-APase) at both the molecular and cellular levels. Lupin plants had increased levels of total acid phosphatase activity within two to five days after P was withered and levels approximately doubled by 15 days. Lateral roots and not main tap roots were responsible for this increase in acid phosphatase activity. Immunoblot analysis using antibodies raised against a purified S-APase showed that the synthesis of this protein was induced as early as 2 days in the P deficient treatment and that levels dramatically increased by 15 days. In contrast, no immunoreactive polypeptide was evident from crude extracts prepared from root tissues of - P treated plants. Immunocytochemical analysis revealed that the protein was located on the surface of epidermal cells of main tap roots and in the cell walls and intercellular spaces of lateral roots and lateral roots may actively secrete S-APase as soon as it is synthesized. A cDNA clone encoding the S-APase was isolated from a cDNA library constructed from lupin roots grown without P. The clone was 2,187 bp in length and had a single open reading frame of 637 amino acid residues. The deduced amino acid sequence was identical to the N-terminal region and peptide sequences of S-APase purified from lupin roots. A hydrophobic signal peptide region consisted of 31 amino acids. The primary structure was highly homologous to iron-zinc purple acid phosphatase from *Phaseolus vulgaris* (76%), secretory purple acid phosphatase from Arabidopsis thaliana (71%), and two *Aspergillus* phosphate repressible acid phosphatases (59% and 58%).

Introduction

Phosphorus is one of the major essential elements required for growth by higher plants as it is required for the synthesis of phospholipids, nucleotides, ATP, glycophosphates, and other phosphate esters. Currently, much of the cultivated lands throughout the world is deficient in available phosphate and this is one of the main factors that limits food production in many countries.

Although plant roots readily absorb inorganic phosphate, most soil phosphate is inaccessible to plants and exists in various chemical forms such as Fe, Al, or Ca phosphate, and organic P. Plants have developed various strategies to liberate phosphorus including the secretion of organic acids, piscidic acids, and secretory acid phosphatase (S-APase) from roots. It has been proposed that S-APases secreted from roots can hydrolyze organic phosphates in the soil liberating inorganic phosphate which can subsequently be absorbed and utilized by the plant. This function of S-APases has been demonstrated in studies with beets and tomatoes (Tadano and Komatsu, 1994). Addition of this enzyme to the soil resulted in improved growth of tomato and beet plants over plants grown in untreated soil. Moreover, the growth of beet plants was even higher in S-APase-supplemented soil than in soil treated with phosphorus fertilizer (Tadano and Komatsu, 1994). These results indicate that plants can liberate bound phosphorus by secretory acid phosphatases.

Plants in general secrete S-APases from their roots under phosphorus-deficient conditions. However, the level and duration of S-APase secretion is dependent on the plant species. We have shown earlier that lupin plants secrete large amounts of S-APase under phosphorus-deficient conditions (Sakai and Tadano, 1993; Tadano and Sakai, 1991; Tadano et al., 1993). We have also reported the purification and some properties of S-APase from lupin roots (Ozawa et al., 1995). The molecular mass of this enzyme was estimated to be 140 kDa by gel filtration chromatography and 72 kDa under denaturing conditions as analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). These results indicate that S-APase probably exists as a homodimer. The

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enzyme had a very high affinity towards its substrate with a K_m value of 0.027 m*M* for *p*-nitrophenylphosphate. The enzyme is optimally active at pH 4.3 although significant enzyme activity is also evident at pH 3.0 to 6.0. Moreover, S-APase is very stable between pH 4.0 to 9.0. Like many other secretory proteins, the S-APase is glycosylated which may confer protection against proteolytic enzymes and confer stability towards a wide range of pHs. These properties, i.e. high substrate affinity, relatively wide pH optimum and temperature stability are consistent with the suggested role of S-APase in generating Pi from organic phosphates present in soils. Furthermore, it has been confirmed by a root-box experiment that S-APase are distributed in rhizosphere within about 2.5 mm from the root surface of lupin and that the amount of organic P in the rhizosphere soil are depleted (Li et al., 1997).

In an effort to understand the mechanism of S-APase synthesis and secretion we have undertaken a study to determine its expression at the molecular and cellular levels.

Materials and methods

Plant materials and cultivation

Lupin (*Lupinus albus* L. cv. Kievskij) seeds were sterilized in 70% (v/v) ethanol for 1 min and then germinated and grown on perlite in a greenhouse. Seven days after germination, the seedlings were transferred to a 56 L plastic vessel containing nutrient solution with or without phosphate (+P or -P treatment). The composition of the nutrient solution was 1.4 m*M* of NH_4NO_3, 2.0 m*M* of $NaNO_3$, 2.0 m*M* of $MgSO_4$, 2.0 m*M* of $CaCl_2$, 1.0 m*M* of K_2SO_4, 36 μM of $FeSO_4$, 17 μM of $MnSO_4$, 3.1 μM of $ZnSO_4$, 0.16 μM of $CuSO_4$, 47 μM of H_3BO_4, and 7.5 n*M* of $(NH_4)_6Mo_7O_{24}$. Phosphate-supplemented nutrient media contained 65 μM NaH_2PO_4. The pH of the solution was daily adjusted to 5.3 for the first 7 days. Three lupin plants were harvested every 2-4 days. The roots were then collected and washed with deionized water. Roots were dried with paper towels and stored at -80°C.

Extraction of crude protein

Lateral roots were homogenized with a Polytron® (Kinematica GmbH) in 50 m*M* Tris-HCl, pH 7.1, containing 1 m*M* 2-mercaptoethanol and 1 m*M* EDTA. The homogenized tissue was filtered through nylon mesh and the filtrate was centrifuged at 15,000 *g* for 10 min. The supernatant (crude extract) was made up in 12% (v/v) glycerol and stored at -20°C.

Measurement of Pi and acid phosphatase activity

Pi (orthophosphate) was measured by the spectrophotometric method of Saheki et al. (1985). Acid phosphatase (APase) activity was assayed by the release of *p*-nitrophenol from *p*-nitrophenylphosphate according to the method of Ueki and Sato (1977). One unit of APase activity is defined as the amount of enzyme that hydrolyzes 1 μmol of p-nitrophenylphosphate per minute.

Preparation of polyclonal antibody against S-APase and western blot analysis

S-APase was purified by the method of Ozawa et al. (1995). The enzyme solution (1 mL; about 1 mg) was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into a rabbit. Rabbits were re-inoculated seven days later. Six days after the second inoculation, serum was collected and then centrifuged to remove coagulated blood cells. Specificity of the antibody was assessed by immunoblot analysis. SDS-PAGE was performed using a Mini-Protean gel system (Bio-Rad Laboratories) according to the method of Laemmli (1970). S-APase was visualized using western blotting detection kit (Amersham) according to the manufacturer's instruction.

Localization of S-APase in root tissues

Twenty days after phosphate treatment, lupin roots were incubated in fixation buffer [4% (w/v) paraformaldehyde, 0.5% (w/v) glutarardehyde, 60 m*M* sucrose and 40 m*M* phosphate; pH 7.4], for 7 hr and then placed in washing buffer (60 m*M* sucrose and 40 m*M* phosphate; pH 7.4). Protein-fixed roots were sectioned 150 mm thick using a Microtome (NK-System). Sectioned root tissues were then incubated in blocking buffer [3% (w/v) BSA in PBS (137 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* $NaH_2PO_4 \cdot 12H_2O$ and 1.5 m*M* KH_2PO_4; pH 7.2)] for 60 min, and then washed in phosphate buffered saline (PBS). The sections were hybridized with anti-S-APase antibody in PBS with 0.1% BSA after the blocking procedure at 4°C overnight. After washing with PBS, the sections were incubated with fluoresceinisothiocyanate-labelled goat anti-rabbit IgG antibody (BioMakor) in PBS with 3% BSA at room temperature for 60 min. The reaction was terminated by washing the sections in PBS. The root sections were then analyzed using a fluorescence microscope.

Partial amino acid sequencing

The N-terminal amino acid sequence of purified S-APase was determined using Pro-Spin™ (Perkin

Elmer Cetus Inc.). Various peptides digested by CNBr or lysyl-endopeptidase were separated by HPLC and analysed using 130A Separation System and 447A Protein Sequencer (Perkin Elmer Cetus Inc.). The N-terminal region, one peptide of CNBr digestion, and four peptides of lysylendopeptidase digestion were sequenced.

Polymerase chain reaction (PCR)

A sense primer was designed from the N-terminal amino acid sequence as 5'-GGITATAATG CICCICAGCAGATICA-3', and an antisense primer from one of the lysyl-endpeptidase digested peptides as 5'-TCGTAIGCGTGIACGTGICCIGCGAAIAC-3'. PCR was performed using the DNA Thermal Cycler PJ 1000 (Perkin Elmer Cetus Inc.) using a cDNA mixture prepared from mRNAs isolated from -P lupin roots as templates. The DNA denaturation step was set at 94°C for 1 min with the primer annealing step at 50°C for 2 min and the primer extension step at 72°C for 3 min. The reaction was allowed to run for 25 or 30 cycles. After the reaction, only one product (about 0.9 kb) was observed by agarose gel electrophoresis.

DNA probe preparation

The PCR product was blunt-ended with T4 DNA polymerase, and then subcloned into the *Eco*RV site of pBluescript II SK+ vector (Stratagene), and sequenced by the dideoxy method (Sanger et al., 1977). This fragment consisted of 932 bp. The deduced amino acid sequence included sequences identical to the amino acid sequences obtained from the proteolytic products of purified S-APase. A portion (491 bp) of this PCR fragment was further amplified by PCR and subcloned into the *Eco*RV site of pBluescript II SK(+) vector as the DNA probe for the S-APase gene. The subcloned plasmid was named pBLAP493, and the *Eco*RI/*Xho*I digested fragment was named LAP493.

Construction of a cDNA library

Total RNA was isolated from -P treated roots using the SDS/phenol method as described by Palmiter (1974). Double-stranded cDNA was synthesized from poly(A)+ RNA purified by OligoTex™-dT30 (Takara Shuzo Co., Ltd.) using a cDNA synthesis kit (Amersham). Blunt-ended double-stranded cDNA was *Eco*RI-methylated prior to ligation with *Eco*RI linkers (5'-pCCGGAATTCCGG-3'; Takara Shuzo). The cDNA molecules containing *Eco*RI cohesive ends were ligated into the *Eco*RI site of λ gt10 phage vector (Stratagene). The recombinant phage were packaged with Gigapack II Gold (Stratagene) and amplified in *Escherichia coli* NM514.

Screening by plaque hybridization

The LAP493 was labeled with digoxygenin (DIG) using a commercial DNA labelling kit (Boehringer Mannheim Biochemica) and used to screen the cDNA library. About 5.5×10^4 recombinant phage of the library were transferred onto duplicateny-lonmembranes (Hybond-N+; Amersham) and then hybridized with the DIG-labelled LAP493 at 60°C for 15 h. Hybridization and detection were performed using a DIG Detection Kit (Boehringer Mannheim Biochemica) according to the manufacturer's instruction.

DNA sequencing

DNA sequencing was performed by the dideoxy chain-terminal method (Sanger et al., 1977) using 7-deaza-dGTP instead of dGTP using a 370A DNA Sequencer (Perkin Elmer Cetus Inc.). Deletion series of the cDNA clone were prepared using a deletion kit for kilo-sequence (Takara Shuzo). Nucleotide and amino acid sequences were analyzed using the Gene/Protein sequence database of a DNASIS (Hitachi) computer. Homology searches were performed with the BLAST program (Altschul et al., 1990; Gish and States, 1993) against the PDB, SWISS-PROT, PIR, and GenPept databases through the National Center for Biotechnology Information. Alignment of the amino acid sequences was performed using the MALIGN program (Hein, 1990) through DDBJ (DNA databank of Japan).

Results and discussion

Induction of acid phosphatase in roots

In previous studies (Ozawa et al., 1995) we showed that roots of lupin plants grown under phosphate-deficient conditions (-P treatment) induced the secretion of S-APase. In order to obtain a better understanding of this phenomenon, we grew lupin plants in the presence and absence of supplemented phosphate and analyzed the roots for Pi and total acid phosphatase (APase) in the main and lateral roots during the first 15 days of treatment (Fig. 1). Under -P treatment, Pi levels in the main and lateral roots quickly decreased within two days (Fig. 1A). Under +P treatment, however, Pi levels did not change significantly for the first two days and then dramatically increased (Fig. 1B). The APase activity levels remained almost constant in the main roots irrespective of whether the plants were grown in the presence or absence of P (Fig. 1C and D). In contrast, the APase activity levels in lateral roots more than doubled by 15 days under -P treatment

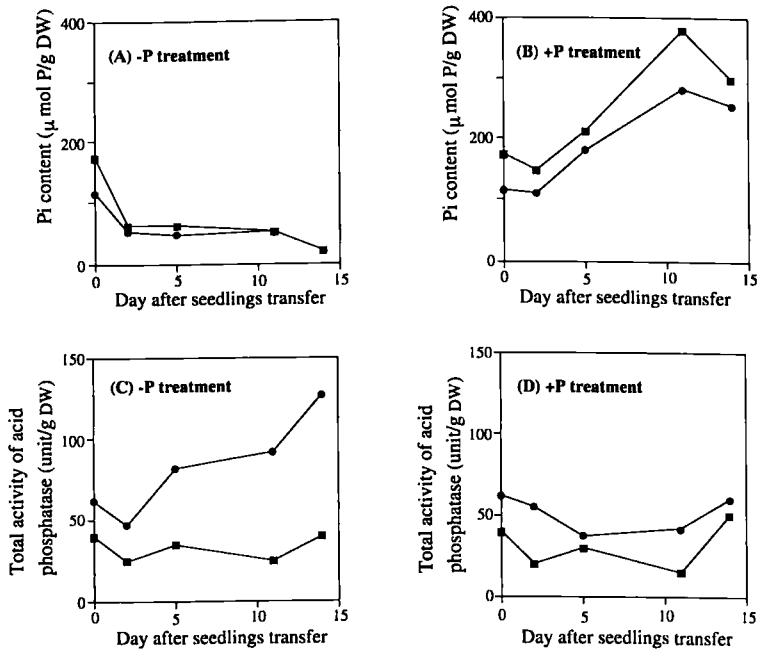

Fig. 1. Effect of phosphorus treatment on phosphate content and total acid phosphatase activity levels of lupin roots. The contents of phosphate and total activities were assayed in lupin roots after plants had been transferred to medium with or without phosphate. Closed squares and circles indicate contents of phosphate and total activities in main and lateral roots, respectively.

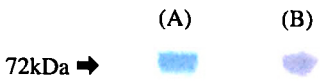

Fig. 2. Assesment of specificity of anti-(S-APase)-IgG. (A), a SDS-PAGE of purified S-APase; (B), immunoblot analysis of crude extract (20 μg) from -P treated roots using anti-(S-APase)-IgG from rabbit.

(Fig. 1C). These results indicate that APase was synthesized specifically by lateral roots and not by main roots under phosphorus-deficient conditions.

Specificity of polyclonal antibody against S-APase

Specificity of the antibody against S-APase was assessed by immunoblot analysis. When crude extracts from lupin roots are resolved by SDS-PAGE and subsequently analyzed by immunoblot techniques, only a single band corresponding purified S-APase is evident (Fig. 2). These results indicate that the antibody specifically recognizes only S-APase which enabled us to directly assess the levels of this protein during -P treatment.

Time course of S-APase synthesis during -P treatment

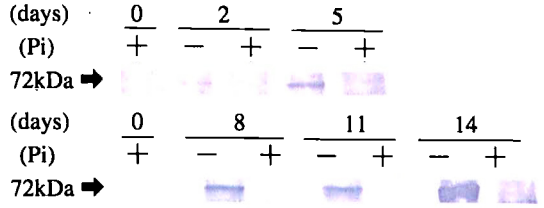

Fig. 3. Time course of lupin S-APase synthesis. Crude extracts after the phosphate treatment were subjected to SDS-PAGE and analyzed by immunoblot using anti-(S-APase)-IgG. The numbers indicate days after the phosphate treatment. + or - indicates treatment with or without phosphate.

Plants subjected to -P and +P treatments were grown up to 14 days and the induction of S-APase synthesis in lateral roots determined by immunoblot analysis. S-APase antigen was first detected after two days of -P treatment and then steadily increased to 14 days (Fig. 3). The S-APase protein was not detected in the lateral roots of +P treated plants thoughout the incubation period. No protein band corresponding to S-APase was detected from the lateral roots of +P treated plants subjected to the same experimental conditions. The results depicted in Figure 3 are consistent with the increase of total APase activity

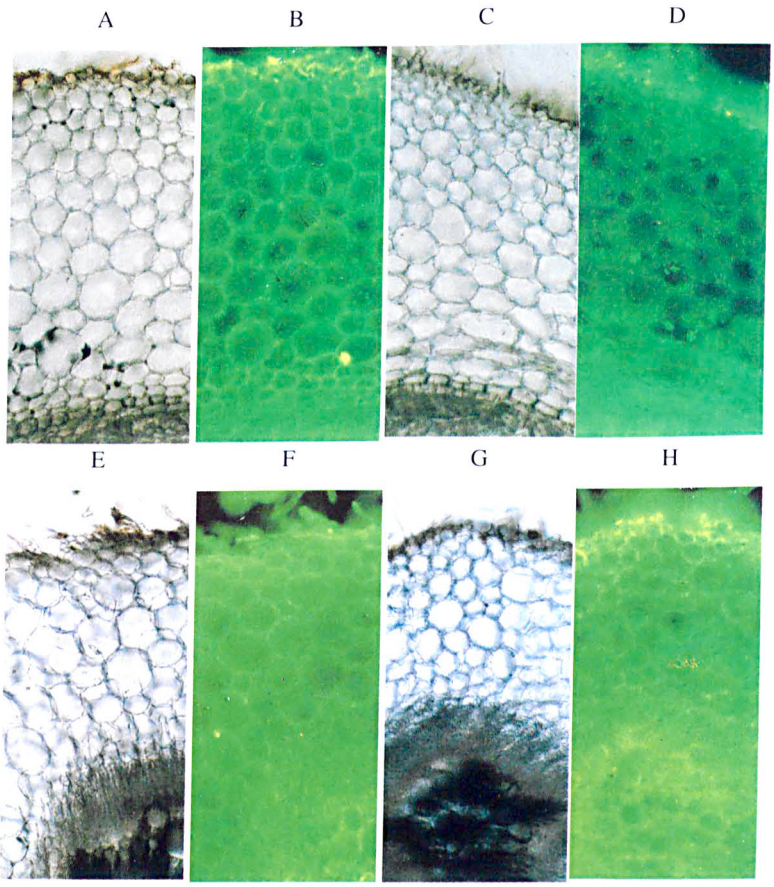

Fig. 4. Localization of S-APase in lupin roots. A to D and E to H indicate the sections of main and lateral roots, respectively. A, C, E, and G, structure of tissue by light microscope; B, D, F, and H, localization of S-APase by fluorescence microscope; A, B, E, and F, the -P treatment; C, D, G, and H, the +P treatment.

exhibited by lateral roots from -P treated plants shown in Figure 1C. Moreover, these results indicate that S-APase is at least one component of the APase activity induced under -P treatment.

The results depicted in Figures 1C and 3, however, are not entirely consistent with one another. In Figure 1C, total APase enzyme activity only increased about two-fold under -P treatment, while in Figure 3 the S-APase protein increased many-fold from a level that could not be detected by immunoblot analysis to a very high readily detectable level. Furthermore, we have shown previously that there are several isozymes of APase in the lupin roots (Ozawa et al., 1995). Assuming that protein levels correspond to enzyme activity levels, these results suggest that S-APase is only one of several APases produced by lateral roots.

Localization of S-APase in root tissues

To investigate the localization of S-APase in lupin roots, sections of main and lateral roots from 20 day old -P treated plants were incubated with anti-S-APase and then tissues examined by fluorescence microscopy. In the main roots, S-APase was localized in the surface of endodermal and epidermal cells (Fig. 4; A to D). This is consistent with the result that APase activity is high in the epidermis of roots under P deficient conditions (Bieleski et al., 1972). A large amount of S-APase was observed both in cell walls and between cells of lateral roots in the -P treatment (Fig. 4 E and F). This result indicated that S-APase was synthesized in all tissues of the lateral roots. However, localization of S-APase observed in lateral roots on the -P treatment was not as clear as compared with that in the main roots. Ozawa et al. (1995) reported that S-APase

activity in -P medium for lupin roots increased after 5 days. Including this result and these in Figures 1 and 3, lateral roots may actively secrete S-APase as soon as it is synthesized. On the other hand, S-APase in the epidermis was secreted less from roots under +P conditions. Future research should examine whether or not the enzyme is secreted just after synthesis.

Isolation and characterization of cDNA clone for S-APase

A cDNA library consisting of about 5.5 x 10^4 recombinant phages was screened with a DIG-labeled LAP493 which resulted in the isolation of five positive clones after the second screening. These clones were plaque-purified and phage DNAs were purified for further analysis. The longest cDNA clone, designated LASAP1, was selected for further study. DNA sequence analysis showed that the DNA fragment of LASAP1 was 2,187 bp in length (data not shown; sequence is prepared for registration to databases). It contained a single long open reading frame of 1,914 nucleotides encoding 637 amino acid residues. A putative polyadenylation signal (5'-AATAAA-3') is located 214 bp downstream from the stop codon.

The deduced primary sequence agreed with previous amino acid sequences determined from proteolytic fragments of the purified S-APase (data not shown). These results indicate that LASAP1 encodes for S-APase from lupin. The N-terminus of the deduced protein contained a 31 amino acid leader sequence that is not present at the N-terminus of the purified S-APase. Consistent with other signal peptides that target the protein to the endomembrane system for ultimate secretion, the leader sequence has a hydrophobic character. This putative signal peptide is typical of signal peptides however, in that it does not have a basic amino acid near the N-terminus.

A homology search was performed between the deduced amino acid sequence and submitted amino acid sequences contained in the PDB, SWISS-PROT, PIR, and GenPept databases. The deduced amino acid sequence of S-APase was homologous to purple APase from two plants [*Phaseolus vulgaris* iron(III)-zinc(II) purple APase (Klabunde et al., 1994) and *Arabidopsis thaliana* secreted purple APase (Patel et al., unpublished data, Genbank accession No. 1218042)] and of two *Aspergillus* phosphate repressible APases (Mullaney et al., 1995; Ullah et al., 1994) at 76%, 71%, 59%, and 58%, respectively. S-APase is most closely related to the purple APase from another legume, *P. vulgaris*.

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