

Purification of the Major Soybean Leaf Acid Phosphatase That Is Increased by Seed-Pod Removal¹

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Fruit removal for 5 weeks after flowering increased acid phosphatase activity 10-fold in soybean (*Glycine max* L. Merr. var Hobbit) leaves compared with normal seed-pod-bearing plants. The major acid phosphatase activity in leaves was purified over 2700-fold, yielding a single polypeptide of 51 kD with a specific activity of 1353 units/mg protein using *p*-nitrophenylphosphate as the substrate. Isoelectric focusing demonstrated that the purified protein co-migrated with a majority of the activity that increased in leaves following seed-pod removal. Immunoblot analysis demonstrated that at least part of the increased activity was due to an increased abundance of the phosphatase protein. In situ enzyme activity staining localized most of the total phosphatase activity to vascular tissues, the leaf paraveinal mesophyll cell layer, and the lower epidermis. This distribution and the response to seed-pod removal paralleled previous results for soybean vegetative storage protein (VSP) α and β . However, in a native polyacrylamide gel the VSP detected by immunological staining of electrophoretically transferred protein did not migrate with the majority of the phosphatase activity. Fractionation of crude leaf protein on concanavalin A-Sepharose yielded a fraction containing 97% of the total VSP but only 0.1% of the total acid phosphatase activity.

Fruit removal from soybean plants substantially alters metabolism in other plant organs. As vegetative organs mature they normally become net exporters of nutrients to plant sinks, such as expanding leaves and developing seeds. An organized degradation of starch, Chl, protein, and other macromolecules contributes to the pool of soluble exports. If seed pods are removed, however, carbon and nitrogen are stored in mature leaves, which accumulate abnormally high levels of starch, Chl, and certain proteins (Wittenbach, 1982).

Two well-characterized vacuolar proteins that are abundant in young organs and in mature organs following removal of seed pods are VSP α and β . These are considered to be storage proteins because of their abundance, their localization in vacuoles, and the regulation of VSP gene expression and corresponding protein level, according to the source-sink status of organs in which they are found. Several other external stimuli, such as high nitrogen levels and jasmonic acid, also induce VSP gene expression (see Staswick, 1990

¹ This is paper No. 10409, Journal Series, Nebraska Agricultural Research Division. Supported by a grant from the U.S. Department of Agriculture Competitive Research Grants Office (90–3762–5532) to P.E.S.

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and 1994, for reviews). Depodding increases the quantity of certain other proteins in leaves (Wittenbach, 1983; Crafts-Brandner et al., 1991), albeit to a lesser extent, and one of these is a lipoxygenase (Tranbarger et al., 1991). Whether any of these should be considered storage proteins is not clear at this time.

A tomato acid phosphatase sequence deduced from a recently isolated cDNA has 44% identity with the soybean (*Glycine max*) VSP polypeptides (Williamson and Colwell, 1991). Physical characteristics of the two proteins are also similar (Aarts et al., 1991), although tomato Aps1 is apparently much less abundant than soybean VSP. VSP α and β were also recently reported to have acid phosphatase activity (DeWald et al., 1992), although the specific activity was very low. There is precedent for abundant storage proteins having enzymic or other biological activity. Examples include some seed lectins and patatin, a storage protein of potato tubers that has lipid acyl hydrolase activity (Andrews et al., 1988). In most cases, including soybean VSP α and β , it has not been demonstrated that the enzymic or other biological activity is a necessary function of these proteins.

Plant acid phosphatases are involved in phosphate metabolism and appear to function in response to phosphate deficiency (Ueki and Sato, 1971; Besford, 1979; Duff et al., 1991). Other suggested functions include involvement in the response to salt stress (Pan, 1987) and water deficit (Barrett-Lennard et al., 1984). However, a comprehensive understanding of the metabolic function of acid phosphatases is lacking, partly because of the heterogeneity and large number of phosphatases, as well as their general lack of substrate specificity (Duff, 1991).

In this study we purified a leaf acid phosphatase that is a major source of increased activity in depodded plants. We also found that VSP could account for only a minor amount of the total activity in soybean leaves from depodded plants.

MATERIALS AND METHODS

VSP Fractionation

Soybeans (*Glycine max* L. Merr. var Hobbit) were grown in a greenhouse, growth chamber, or in the field as indicated for each experiment. Seed pods were removed from plants as previously described (Staswick, 1989). For isolation of VSP, frozen (–80°C) leaves were ground in 25 mM Tris-HCl (pH

Abbreviations: Aps1, tomato acid phosphatase-1; pNPP, *p*-nitrophenylphosphate; VSP, soybean vegetative storage protein.

7.6), 1 mM EDTA, 0.1 mM PMSF, 5 mM DTT. The crude extract was fractionated by ammonium sulfate precipitation and Con A-Sepharose (Sigma) chromatography as described by Rapp et al. (1990). All steps after tissue collection were performed at 4°C.

Purification of an Acid Phosphatase

Leaves were harvested from plants grown in a growth chamber and depodded twice weekly for 5 weeks after flowering. Leaves were stored frozen at -80°C. Frozen tissue wrapped in foil was broken by hand, then 20 g was homogenized with a Polytron at 4°C in 150 mL of 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 30 mM ascorbic acid, 1 mM DTT, and 3% (w/v) insoluble polyvinylpyrrolidone. After filtration through four layers of cheesecloth, the extract was centrifuged at 17,000g for 10 min. The supernatant was brought to 2 M ammonium sulfate and stirred on ice for 1 h. The supernatant was recovered after centrifugation as before and brought to 2.8 M with ammonium sulfate. After 1 h on ice the resulting precipitate was collected by centrifugation as described above. The pellet was resuspended in 5 mL of 25 mM Mes, pH 6.5, insoluble material was removed by centrifugation (5 min at 17,000g), and the supernatant was desalted by gel filtration chromatography on Sephadex G-25 (1.5 × 27 cm) in the same buffer. Precipitate that formed during desalting was removed by centrifugation.

The supernatant was loaded on a DEAE Sephadex A-50 column (2.5 × 10 cm) equilibrated with 25 mM Mes, pH 6.5. Typically the first 20 mL contained the majority of acid phosphatase activity, which did not bind to the column. This fraction was centrifuged as before and then loaded directly on a hydroxylapatite column (1 × 18 cm) equilibrated with 10 mM Na phosphate, pH 7.3. The column was eluted with a 200-mL linear gradient of Na phosphate (from 10–400 mM, pH 6.7). The single activity peak was dialyzed against 4 L of 10 mM NaPO₄ (pH 7) and loaded onto a CM Sephadex C-50 column (0.8 × 30 cm), which was developed with a linear gradient of NaCl to 0.2 M. NaN₃ (0.2%, w/v) was included in all chromatography buffers as an antimicrobial agent. All steps beginning with tissue homogenization were done at 4°C, with the exception of hydroxylapatite chromatography, which was conducted at room temperature to avoid precipitation of Na phosphate.

Enzyme Activity Assays, Gel Electrophoresis, and Immunological Methods

Total protein in extracts was determined with a modified Bradford assay (Bio-Rad) according to the manufacturer's directions. Phosphatase activity in extracts was determined as described by Ullah and Gibson (1988) in 10 mM Na acetate (pH 5.0), 1 mM pNPP, except that incubations were performed at 25°C rather than 60°C. Standard curves were determined with *p*-nitrophenol (Sigma) for each assay. The optimum pH for the purified phosphatase was determined in 0.15 M Na acetate (pH 4.0–6.6) or 0.15 M Mops (pH 6.0–8.0). The specific activity for each purification step was determined in 0.15 M Mops, pH 6.4. One unit of activity equals release of 1 μmol of *p*-nitrophenol/min at 25°C.

Nondenaturing polyacrylamide gels were prepared and stained as described by Aarts et al. (1991). IEF gels contained 7.5% (w/v) acrylamide, 0.2% (w/v) N,N'-methylenebisacrylamide, 6 M deionized urea, and ampholytes (Fisher) at pH 3 to 10 (0.018%, v/v) and pH 7 to 9 (0.018%, v/v). IEF was performed at 5.5 W for 5 to 7 h at 4°C. Gels were incubated for 1 h in two changes of 50 mM Na acetate, pH 5.5, 10 mM MgCl₂, and then stained as described for nondenaturing gels. Electrophoretic transfer of protein from nondenaturing gels was done after incubating gels for 1 h at 25°C in 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 2.5% (v/v) 2-mercaptoethanol. Standard SDS-PAGE, immunoblotting, and silver staining procedures were as previously described (Staswick, 1989). Antisera against the purified acid phosphatase was prepared as described previously (Staswick, 1988). Prior to processing immunoblots to detect phosphatase, protein were treated to oxidize the antigenic glycosylated side chains (Laine and Faye, 1988). Blots were incubated for 2 h at room temperature in 100 mM Na acetate (pH 4.5), 10 mM Na metaperiodate followed by incubation for 30 min in 10 mM potassium phosphate, pH 7.4, 15 mM NaCl, 50 mM Na borohydride.

Phosphatase Activity Staining in Situ

Histochemical localization of acid phosphatase activity was done by modification of procedures previously described (Davenport, 1960; Pylotis et al., 1979; Gabard and Jones, 1986). Tissue was hand-sectioned with a razor blade over water and sections were immediately transferred to 3.6 mM PbNO₃, 100 mM Na acetate, pH 5.8, 50 mM Suc (buffer A) on ice until sectioning was completed. Sections were then incubated with substrate by transferring them to buffer A containing 3 mM pNPP for 1 h. Phosphate released from the substrate formed a Pb phosphate complex. Sections were then rinsed four times in buffer A lacking pNPP and PbNO₃. Sections were fixed in 33% acetic acid in ethanol (v/v) for about 30 min to remove Chl and then equilibrated in 100% ethanol for about 1 h. Complexes of Pb phosphate were converted to PbI₂ by incubating sections in a saturated solution of KI in ethanol for 15 min. PbI₂ formed a yellow precipitate that was visualized by dark-field microscopy after sections were mounted and cleared with venetian turpentine (25% [v/v] propionic acid, 36% [v/v] phenol, 18% [v/v] venice turpentine, 7% [v/v] acetic acid, 14% [v/v] water).

RESULTS

Seed-Pod Removal Elevates Leaf Acid Phosphatase Activity

We examined the effect of seed-pod removal on leaf acid phosphatase activity. Total specific activity was determined in crude leaf extracts using pNPP as the substrate. Figure 1 shows that removing seed pods increased enzyme activity in leaves up to 10-fold over an 8- or 9-week period compared with plants bearing seed pods. This was true of both greenhouse- and field-grown plants. The overall pattern of increased enzyme activity following depodding was very similar to that seen previously for the increase in VSP and its mRNA (Staswick, 1989). The increase in phosphatase activity at 11 weeks in greenhouse-grown, pod-bearing plants also

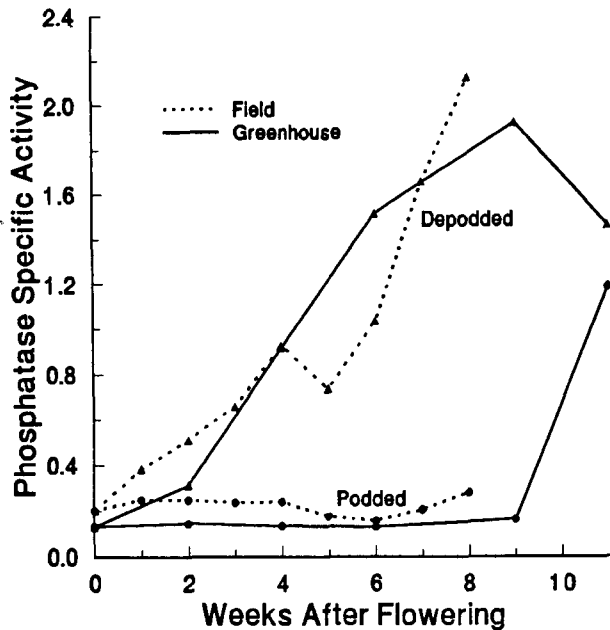


Figure 1. Acid phosphatase activity in leaf protein extracts. Two separate experiments, one with field-grown plants and the other with plants grown in a greenhouse, are shown. Leaves were from the middle two nodes of plants. Enzyme activity was assayed with 10 mM pNPP at 30°C for 45 min and is expressed as $\mu\text{mol pNP released min}^{-1} \mu\text{g}^{-1}$ protein. ▲, Depodded plants; ●, pod-bearing plants.

paralleled the increase in VSP at this time noted previously (Staswick, 1989). This may have resulted because seeds were fully mature and no longer constituted a sink for nutrient mobilization.

Phosphatase Activity Is Localized in Vascular and Epidermal Tissue

The cellular location of acid phosphatase activity was determined by histochemical staining of tissue sections. Tissue was incubated with pNPP substrate, and the product was visualized following formation of a yellow lead iodide precipitate. Representative tissue sections are shown in Figure 2. Activity was notably higher in leaf and stem sections (B and G, respectively) from depodded plants, although the same general distribution of activity occurred in pod-bearing plants (A and F). Most of the activity was limited to the vascular and epidermal regions. Staining was evident in leaf paraveinal mesophyll cells, which are considered an extension of the bundle sheath system. This distribution is similar to that found for VSP (Franceschi et al., 1983; Staswick, 1990) and its corresponding mRNA (Huang et al., 1991). In the absence of substrate (C) or following heating (D) to inactivate enzyme, staining was not detected.

A distinct pattern of alternating stained and unstained regions was found in stems (Fig. 2, F and G). The outer cortical layer, secondary phloem, and primary xylem showed activity, whereas the primary phloem and secondary xylem between them, as well as the pith region, had essentially no

activity. Seed pods (Fig. 2E) also exhibited dense staining in vascular regions, including the major vascular bundles of the dorsal and ventral sutures of the carpel, and the minor veins of the pod wall. Staining was also prominent at the inner and outer epidermal regions of the pod wall.

Purification of a Leaf Acid Phosphatase

To characterize the enzyme(s) responsible for increased acid phosphatase activity in leaves of depodded plants, we purified a protein accounting for the major portion of this activity. A fraction that pelleted between 2.0 to 2.8 M ammonium sulfate was passed over a DEAE Sephadex A-50 anion-exchange column. Most of the phosphatase activity was not retained by the column, although a minor portion eluted after development with a NaCl gradient. The unbound activity was subsequently chromatographed on a hydroxylapatite column and eluted with a NaPO_4 concentration gradient. The major activity peak was recovered and chromatographed on a CM Sephadex C-50 column and eluted with a concentration gradient of NaCl. Contaminants were removed by a second passage over the CM Sephadex column.

Details of the purification and recovery are summarized in Table I. The protein was purified 2700-fold, and we recovered a specific activity of about 1350 units/mg protein. The relative purity of the fractions was judged by silver staining following SDS-PAGE, as shown in Figure 3A. The subunit size of the purified phosphatase was about 51 kD (lane 4).

IEF of Phosphatase

The relative contribution of the purified phosphatase to the total activity in leaves was evaluated by activity staining following IEF. Crude extract from leaves yielded several stained bands, although much of the activity was present in the dark bands labeled 1 through 4 in Figure 3B. These bands constituted the major activity in leaves from depodded (lane DP) and pod-bearing (lane P) plants, and in leaves prior to flowering (not shown). The purified phosphatase activity (lane AP) co-migrated with bands 1, 2, and 3 and with two bands immediately above band 1 that were of lower staining intensity in leaf extracts. Only weak staining was evident for band 4 in the purified sample. For the purified sample, staining was not detected in regions corresponding to other bands from the crude extract, even when 3 times more purified phosphatase was loaded (not shown). The less-intense bands evident in the crude leaf material presumably come from other acid phosphatases or from electrophoretic variants of the protein we purified. These results also demonstrate that in addition to pNPP, the purified phosphatase is also active on β -naphthylphosphate. The purified phosphatase constitutes a major portion of the total activity in soybean leaves, and this activity increases following depodding.

pH Optimum of the Purified Acid Phosphatase

Assay of enzyme activity over a pH range is shown in Figure 4, which demonstrates a pH optimum of about 6.0 in 0.15 M Na acetate and about 6.4 in 0.15 M Mops. We verified

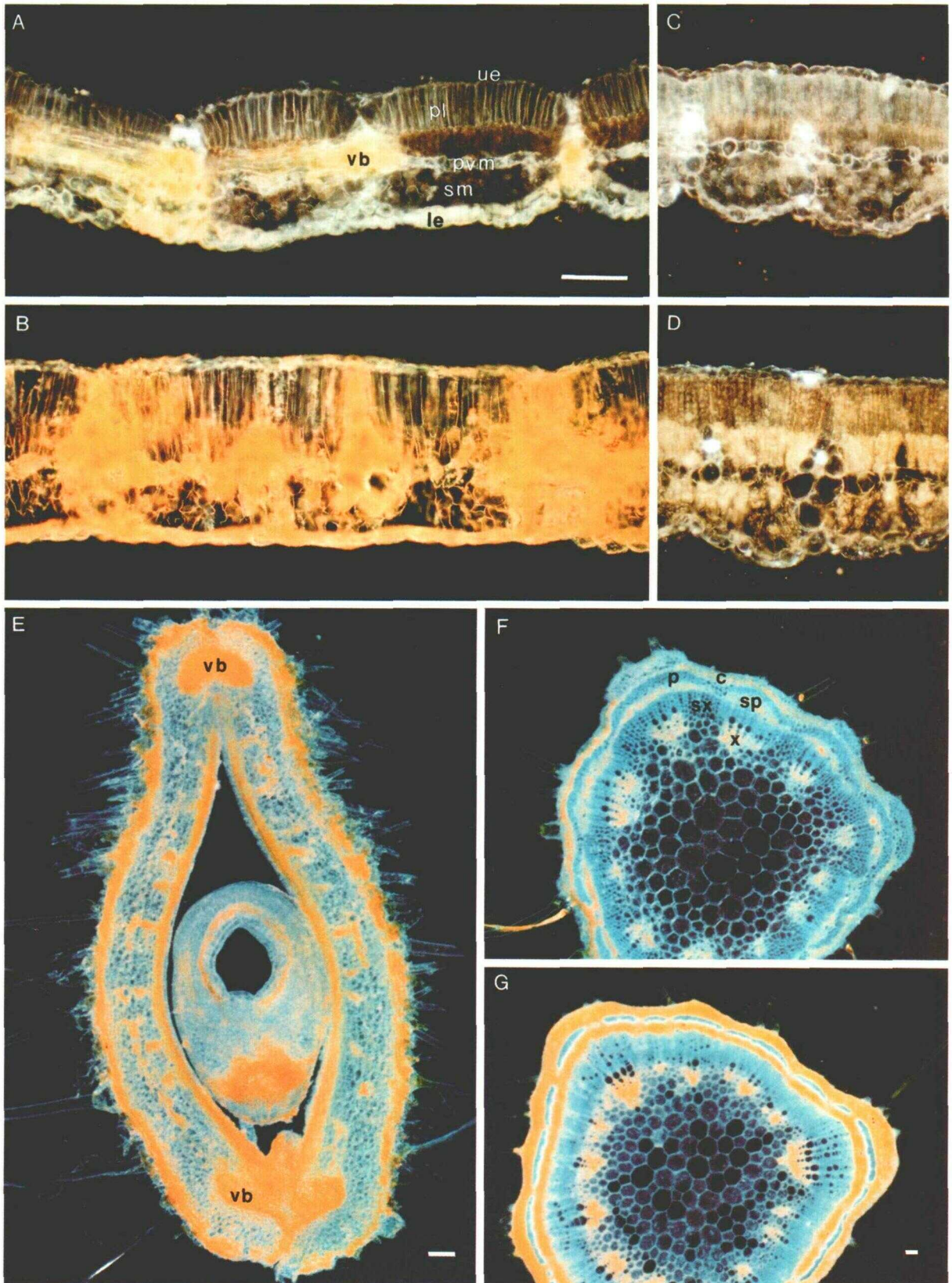


Figure 2. Determination of cellular location of acid phosphatase activity by in situ activity staining. A, Leaf section from a pod-bearing plant at 5 weeks after flowering; B, same as A except seed pods were removed for 5 weeks; C and D, controls that were the same as B except that substrate (pNPP) was not added (C) or the section was heated to 90°C (D)

Table I. Purification of acid phosphatase

Step	Total Activity	Specific Activity	Fold Purification	Yield
	units $\times 10^{-3a}$	units/mg		%
Crude extract	844	0.5		
NH ₄ SO ₄ (2.0–2.8 M) pellet	628	4.0	8	74
DEAE Sephadex	225	7.8	15.6	27
Hydroxylapatite	84	31.0	62	10
CM Sephadex (first)	55	407.0	814	6.5
CM Sephadex (second)	47	1353.0	2706	5.6

^a Units = μ mol *p*-nitrophenol released per min at 25°C.

that the pH of the reactions did not change during the assay by pH analysis of identical samples prior to the addition of NaOH to stop the reaction.

Immunological Detection of Phosphatase in Leaf Extracts

Antisera raised against the purified phosphatase was used to determine its relative abundance in crude leaf extracts. Initial experiments showed that the antisera cross-reacted with several leaf proteins (not shown). Since the phosphatase used to elicit an antigenic response was glycosylated, we assumed that this result was due to the presence of antibodies that recognized common carbohydrate epitopes in other proteins. This was confirmed by first oxidizing carbohydrates after protein transfer to nitrocellulose (Laine and Faye, 1988). This treatment eliminated staining of most protein bands. Figure 5 illustrates that the major protein stained in crude leaf extracts has the same mobility on SDS-PAGE as the purified acid phosphatase. A weakly staining protein of somewhat higher mobility was also detected in both the extract and purified protein sample. This may be a breakdown product of the phosphatase or a minor contaminant of the original phosphatase preparation.

It is evident from Figure 5 that the relative abundance of the phosphatase increases in response to depodding. Comparison of the staining intensity with a dilution series of purified protein indicated that after 9 weeks of depodding the phosphatase was about 0.2% of the total protein, which was about 5-fold higher than in plants at flowering or those bearing seed pods (not shown). This result demonstrates that at least part of the increased acid phosphatase activity in leaves of depodded soybean plants is due to the increased polypeptide abundance of this enzyme.

VSP Accounts for Little of the Total Leaf Acid Phosphatase Activity

VSP α and β are abundant and were recently reported to have acid phosphatase activity (DeWald et al., 1992). Therefore, we investigated the contribution of VSP to the total

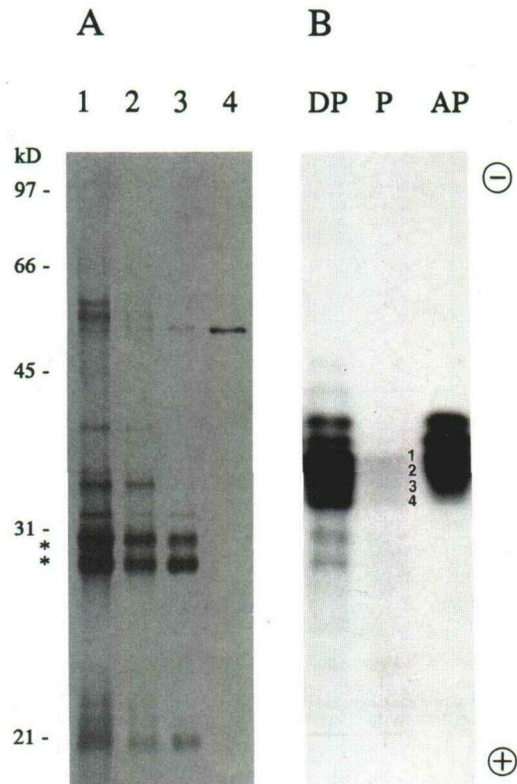


Figure 3. Gel electrophoresis of purified acid phosphatase. A, Protein from each purification step was analyzed by silver staining after SDS-PAGE. Lane 1, NH₄SO₄ fraction (2.6 μ g); lane 2, DEAE Sephadex chromatography (1.6 μ g); lane 3, hydroxylapatite chromatography (1 μ g); lane 4, CM Sephadex chromatography (0.05 μ g). The mobility of molecular mass markers is indicated to the left. Asterisks denote the position of VSP polypeptides. B, IEF of purified acid phosphatase and total leaf extracts. DP, Depodded plant (20 μ g); P, podded plant (20 μ g); AP, purified protein (0.01 μ g).

activity in leaves from depodded soybean plants. Duplicate lanes of a native polyacrylamide gel were either stained for enzyme activity or immunoblotted and stained using VSP antiserum. Gel portions and immunoblots were aligned after staining using holes punched in each gel immediately after electrophoresis. These spanned the full length of the gels. Figure 6 confirms that the specific acid phosphatase activity increased markedly following depodding. It is also evident that VSP did not migrate with the majority of the activity, even though VSP comprised about 40% of the total leaf protein from depodded plants at this stage (Wittenbach, 1983).

The contribution of VSP to the total acid phosphatase was further examined by fractionation of crude extract by Con A-Sepharose column chromatography. The elution profile and

(Continued from previous page) to inactivate enzyme before substrate addition; E, young seed pod sectioned through a developing seed. F and G are stem sections from a pod-bearing and a depodded plant, respectively. Staining intensity is comparable among similar organs but not between different organs. Scale bars indicate 1 mm. A through D are to the same scale, as are E and F. c, Cortex; le, lower epidermis; p, primary phloem; pl, palisade layers; pvm, paraveinal mesophyll; sm, spongy mesophyll; sp, secondary phloem; sx, secondary xylem; ue, upper epidermis; vb, vascular bundle; x, xylem. Photography was done using dark-field illumination; a blue filter was used for E, F, and G.

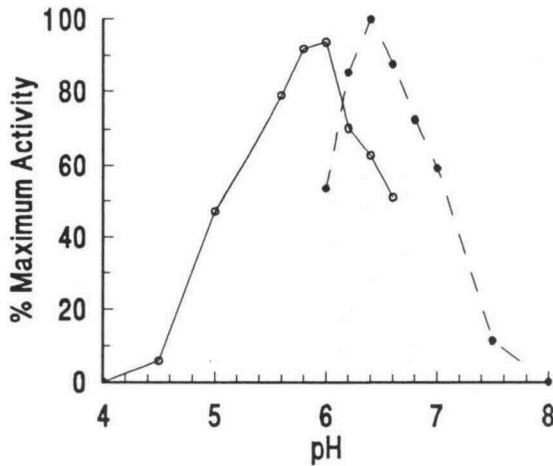


Figure 4. Optimal pH for activity of purified acid phosphatase. Values are the mean of triplicate samples for each pH tested and are expressed as a percentage of maximal activity. Assays were done in 0.15 M Mops (- - -) or 0.15 M Na acetate (—).

associated phosphatase activity is shown in Figure 7. Nearly all of the activity was bound to the column and subsequently eluted with methyl glucopyranoside (fractions 22–24). Enzyme activity was determined on an equal volume basis for each fraction and is expressed here on a logarithmic scale. Summing the activity in each fraction revealed that 99.9% of the total recovered activity was in peak 3, the material eluted with methyl glucopyranoside (note that activity is expressed on a logarithmic scale in Fig. 7).

Con A-Sepharose fractions were analyzed by SDS-PAGE and the results are shown in the photograph at the center of Figure 7. Although VSP is a glycoprotein, the majority did not bind tightly to Con A-Sepharose but was purified to near homogeneity in the "tail" (peak 2) of the unbound material (asterisks). This result is consistent with an earlier study that found that a portion of the VSP interacted weakly with Con

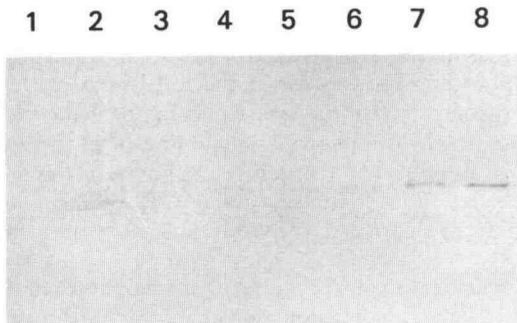


Figure 5. Immunoblot of acid phosphatase in leaf extracts. Plants were treated as in Figure 1. Leaves were harvested at flowering (lane 1) or at 2, 4, or 9 weeks after flowering from pod-bearing (lanes 2, 3, and 4, respectively) or depodded (lanes 5, 6, and 7, respectively) plants. Lane 8 was loaded with purified 51-kD acid phosphatase (25 ng); all others were loaded with total leaf soluble protein (6 μ g). Membranes were treated to oxidize carbohydrates as described in the text.

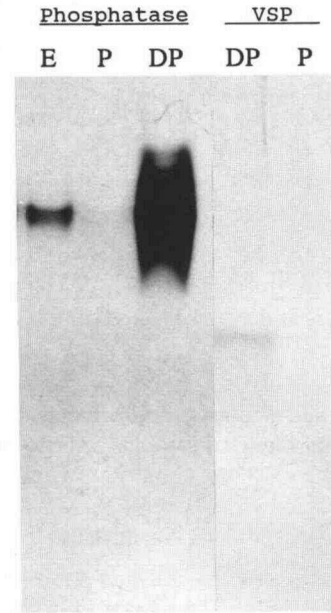


Figure 6. Electrophoretic mobility of leaf acid phosphatase activity and VSP. Crude protein was analyzed by nondenaturing PAGE. Three lanes on the left were stained for phosphatase activity. Two lanes on the right were denatured in SDS, transferred to nitrocellulose, and protein was detected with soybean VSP antisera. Each lane was loaded with 15 μ g of protein. E, Expanding leaf; P and DP, leaves from podded and depodded plants, respectively, 6 weeks after flowering.

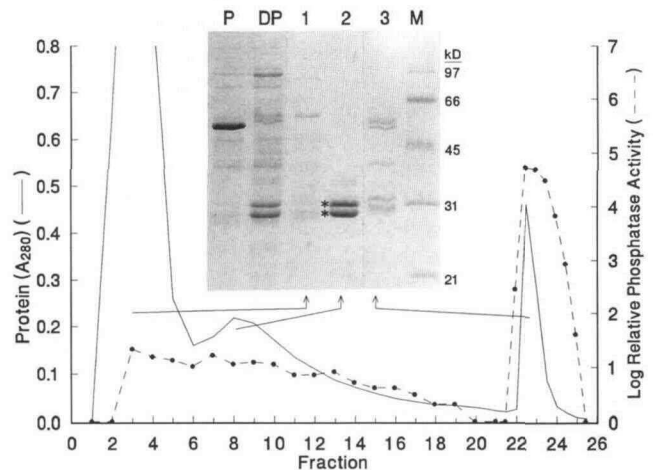


Figure 7. Chromatography of phosphatase activity on Con A. A 2.0 to 2.8 M NH_4SO_4 fractionation of leaf protein from depodded plants was applied to the column. Elution with 50 mM methyl glucopyranoside was begun with fraction 22. Activity was assayed with pNPP as substrate for each fraction and is plotted in arbitrary units on a log scale. Peak 1, Fractions 1–6; peak 2, fractions 7–21; peak 3, fractions 22–25. Aliquots from each peak were analyzed by SDS-PAGE and stained with Coomassie blue (photograph at center). Lanes P and DP were loaded with total leaf extracts from podded and depodded plants, respectively. Lane M, Molecular mass markers. VSP α and β migrate at about 27 and 29 kD, respectively, and are denoted with asterisks.

A-Sepharose and eluted slowly during the initial wash (Rapp et al., 1990). We estimated the relative amount of VSP in each peak by western blotting of appropriate dilutions from each peak and subsequent immunodetection with VSP antisera. Only about 3% of the VSP recovered from the column was present in peak 3.

We could not determine whether any of the acid phosphatase activity in peaks 1 and 2 was due to VSP, since minor amounts of other phosphatases may have been present. The fact that peak 1 contained little VSP compared with peak 2, but that both had about the same amount of activity suggests that at least for peak 1, much of the activity was not due to VSP. In any case, no more than 0.1% of the total acid phosphatase activity recovered from the column was associated with the fractions containing 97% of the VSP (peaks 1 and 2).

The low activity of the fractions containing VSP was confirmed by comparing the specific activity of peaks 2 and 3. The activities were determined from six independent protein isolations and were variable for peak 2, possibly due to low and varying amounts of other phosphatases in this peak. However, the specific activity of peak 2 was always ≤ 0.1 unit/mg protein and ranged from 200- to 950-fold less than peak 3. We eliminated the possibility that peaks 1 and 2 contained a general inhibitor of enzyme activity by mixing a 10-fold volume excess of both in an assay with peak 3. No change in the activity of peak 3 resulted (not shown).

Several proteins remained bound to the Con A-Sepharose column after extensive washing, including about 3% of the total VSP, and these were eluted with methyl glucopyranoside. In the previous report describing the phosphatase activity of VSP, one purification step was elution from Con A-Sepharose with glucopyranoside (DeWald et al., 1992). Therefore, we considered the possibility that alternate forms of VSP exist: an enzymically inactive form that interacts only weakly with Con A-Sepharose, and an active form with high affinity for Con A-Sepharose. To test this, we loaded peak 2, the nearly inactive fraction containing mostly VSP, on the Con A column a second time. Seventy-seven percent of the protein did not bind to the column the second time, whereas 23% was bound and then eluted with methyl glucopyranoside (not shown). Both the bound and unbound fractions had low phosphatase specific activity, similar to that found previously for peak 2 (Fig. 7). Analysis of fractions from this column by SDS-PAGE indicated that the protein compositions were essentially the same, containing almost exclusively VSP (not shown). This indicates that differential VSP interaction with Con A-Sepharose was not related to different enzymic activity states of VSP.

DISCUSSION

From leaves of depodded soybean plants we have purified an acid phosphatase nearly 3000-fold and recovered a specific activity of about 1350 units/mg protein. This is comparable with the specific activities reported for several other plant acid phosphatases (Duff et al., 1989) using pNPP as the substrate. The phosphatase was also active on β -naphthylphosphate as indicated by staining following IEF. In contrast, we found that the nearly pure VSP fraction from

the Con A-Sepharose column had a specific activity of about 0.1 unit/mg protein, over 10,000-fold lower. The specific activities reported by DeWald et al. (1992) for VSP ranged from 0.3 to 10 units/mg, depending on whether the protein was a hetero- or homodimer of the α and β subunits. It is difficult to directly compare their values with ours because of slightly different assay conditions, including the use of different substrates.

It should also be noted that the VSP dimers used for specific activity determinations by the previous workers came from two distinct tissues; the $\alpha\alpha$ dimer from leaves and the $\alpha\beta$ and $\beta\beta$ dimers from seedling hypocotyls. DeWald et al. (1992) suggested that there are isoforms of the VSP dimers that differ in their specific activities. Thus, it is possible that tissues vary in the isoforms they contain. Interestingly, the specific activity of the $\alpha\alpha$ dimer from leaves was 10- to 20-fold lower than that of the other two dimers, which originated from hypocotyls. Although not quite homogeneous, the specific activity we determined for the mixture of VSP dimers from leaves was very similar to that of the $\alpha\alpha$ dimer, also from leaves. Whether organ or tissue variability in VSP phosphatase activity exists will require additional investigation.

We found that the 51-kD phosphatase accounts for the major fraction of the total leaf activity from depodded plants, as judged by activity staining following IEF. It is apparently a glycoprotein, as are many acid phosphatases, since it was present in the fraction from crude leaf extract that was bound to Con A-Sepharose (not shown).

In addition to the VSP polypeptides at around 27 and 29 kD, Wittenbach (1983) reported that proteins of 54 and 80 kD accumulated in depodded plants. The larger of these is now known to be a lipoxigenase (Tranbarger et al., 1991). A 53-kD acid phosphatase (Ullah and Gibson, 1988) and a 54-kD phytase (Gibson and Ullah, 1988) have been isolated from germinated soybean cotyledons, but it is not known if either of these correspond to the relatively abundant 54-kD protein identified by Wittenbach. The 51-kD acid phosphatase we purified does not co-migrate with the 54-kD protein from depodded plants, which is also more abundant than our phosphatase (not shown).

We also do not know if the phosphatase we isolated is the same as the one previously purified from soybean (Ullah and Gibson, 1988). The reported specific activity for that protein with pNPP was about 10-fold lower than the specific activity we found. This is in spite of the fact that their assay was done at 60°C, the optimum for their enzyme, whereas our assay was done at 25°C. The pH optimum for our enzyme in acetate buffer was also higher: pH 6.0 compared with pH 5.0 (Ullah and Gibson, 1988). The pH optimum reported for VSP was between 5.0 and 5.5 (DeWald et al., 1992), which suggests that we have isolated a unique soybean acid phosphatase.

The purified phosphatase accounts for a major portion of the total activity that increased 10-fold in leaves following depodding. At least part of this increased activity is due to increased protein abundance, not simply enzyme activation. This suggests that gene expression may also be increased, as is true for the VSP genes. The function of this increased activity is unclear. Elevated phosphatase activity under phosphate deficiency or water deficit led to earlier suggestions

that the enzyme(s) functions to alter phosphate metabolism in response to stress (Barret-Lennard et al., 1984; Duff et al., 1991). Although it is not clear how seed-pod removal relates to other plant stresses, this treatment dramatically alters soybean leaf metabolism. Whereas mature soybean leaves eventually catabolize macromolecules and export the resulting products to developing sinks, protein, starch, and Chl are retained at high levels for many weeks following sink removal (Wittenbach, 1982).

Interestingly, we found that soybean leaf acid phosphatase activity was particularly abundant in vascular regions and in paraveinal mesophyll and epidermal cells of leaves. These tissues are also the location of a majority of VSP. Activity was also present in the vascular regions of stems and seed pods, which are also sites of VSP gene expression. Curiously, activity in stems was associated with the secondary phloem and primary xylem, but not the primary phloem or secondary xylem. The reason for this differential distribution is unknown. Phosphatase activity was previously found to be localized in vascular bundles of corn leaves, but a function has not been established (Evert et al., 1988).

The presence of VSP in and near vascular transport tissues has been rationalized as a logical site to temporarily store surplus metabolites (Franceschi et al., 1983). The elevated amounts of acid phosphatase in these same tissues may be used in leaves of depodded plants to convert phosphates from a mobile to a storage form. Alternatively, the acid phosphatase activity may be stored for later use. Stored phosphate from organic molecules may be released at a later time for remobilization to new plant sinks. If this is true, then the stored phosphatase might be inactive *in situ* or sequestered from its substrates until needed for remobilization.

We do not know whether the phosphatase we purified is localized in vacuoles, as is VSP. However, acid phosphatase activity has been demonstrated in the vacuoles of soybean protoplasts (Record and Griffing, 1988), and the low pH optimum of this class of phosphatases has led to the general assumption that they are one of the many hydrolases active in the acidic vacuolar environment (Nishimura and Beevers, 1978).

The fact that VSP α and β were reported to be acid phosphatases (DeWald et al., 1992) led us to examine the relative contribution VSP makes to the total leaf acid phosphatase activity. Surprisingly, despite the extreme abundance of VSP in leaves from depodded plants, it contributed no more than 0.1% to the total leaf acid phosphatase activity. This was supported by native PAGE of crude leaf extract, from which we could detect no activity corresponding to the region of the gel where VSP migrated. Because the VSP fraction we isolated on Con A-Sepharose still contained minor contaminants (Fig. 7, lane 2), we could not determine whether the low specific activity we measured was due entirely to VSP or if another phosphatase contributed. However, our results are consistent with previous findings that VSP has rather low specific activity compared with certain other leaf acid phosphatases (DeWald et al., 1992), and in particular, compared with the 51-kD phosphatase described here.

Further evidence that VSP contributes only a minor amount to the total leaf phosphatase activity comes from the

fact that activity remains essentially constant in plants producing seeds (Fig. 1). In contrast, Wittenbach (1983) reported that VSP declines 6- to 15-fold during this time. If VSP were a major source of activity in leaves, one would expect phosphatase activity to decline during seed development.

The high level of homology between tomato Aps1 and soybean VSP α and β indicate that they derive from a common progenitor. Therefore, it is unlikely that these soybean proteins have some other unknown enzymic activity or function. If high enzymic activity were found for VSP, it might suggest that their role in storage was ancillary to their enzymic function. This does not seem to be the case, although we cannot rule out the possibility that VSP is substantially more active as a phosphatase on an unidentified substrate. DeWald et al. (1992) reported that VSP acted on polyphosphates, but the specific activities were low and about the same as for several other substrates tested. If phosphatase activity is an important function for VSP, it is perplexing why soybean would need such an abundance of this enzyme. The low activity of VSP α and β is consistent with the view that their primary function is in storage.

Our findings may provide additional insight into the origin of some storage proteins. Certain other proteins classified as storage reserves also have various biological activities. These include lectins and protease inhibitors of seeds, and patatin, an abundant potato tuber protein with acyl hydrolase activity. It is not clear that in most cases the high levels of these proteins are necessary or even beneficial because of the activities they exhibit. One hypothesis from this observation is that some storage protein genes are derived from genes coding for enzymes or other biologically active proteins. Duplicate copies of these may have acquired strong developmental and/or tissue-specific promoters that confer the regulatory properties necessary for a storage function. They may also have lost some or all of their original activity, if it was not needed or was supplied by another gene. This may be the case for VSP in soybean.

Only one plant acid phosphatase gene has been isolated; the tomato Aps1 gene, which is related to soybean VSP (Williamson and Colwell, 1991). The dearth of cloned plant phosphatase genes is surprising considering that numerous phosphatases have been studied and purified in a variety of plant species. Furthermore, some phosphatase activities are altered in response to environmental stress, and regulation is at least partially at the level of protein abundance for the response to Pi deficiency (Duff et al., 1991). Our purified protein will be useful to obtain DNA clones for the corresponding gene(s). In addition to depodding, it will be important to establish whether wounding, water deficit, surplus nitrogen, and jasmonic acid induce acid phosphatase gene expression as these stimuli do for soybean VSP. Ultimately, this will contribute to our understanding of acid phosphatase function in plants.

ACKNOWLEDGMENT

The authors acknowledge valuable discussion and advice from Dr. Stephen Duff during this study.

Received August 4, 1993; accepted September 16, 1993.
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