Phosphate Starvation Inducible Metabolism in
Lycopersicon esculentum

II. CHARACTERIZATION OF THE PHOSPHATE STARVATION INDUCIBLE-EXCRETED ACID
PHOSPHATASE

Received for publication October 14, 1987 and in revised form March 17, 1988

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Abstract

Three-day-old suspension cultured cells of Lycopersicon esculentum transf erred to a Pi-depleted medium had 2.7 times the excreted acid phosphatase (Apase) activity of cells transferred to a Pi-sufficient medium. Cell growth during this time period was identical for the two treatments. Excreted Apase activity was resolved into two fractions on a Sephadex G-150 column. Most of the phosphate starvation inducible (psi) enhancement in activity was in the lower molecular weight fraction. These two fractions exhibited different substrate versus pH activity profiles. With a native polyacrylamide gel electrophoresis assay, the lower molecular weight fraction resolved into two bands of activity. Both column fractions resolved into the same single band of activity with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The apparent molecular weight of this enzyme was 57 kilodalton. These data indicate that L. esculentum has at least two isozymes of the psi-excreted Apase and that these isozymes may associate to form high molecular weight aggregates. Labeling studies using [35S]methionine show that the psi response in tomato cells is complex and involves changes in the steady state levels of several excreted proteins.

Enzyme activity was mainly extracellular with the heaviest concentration in corner spaces between the epidermal and hypodermal layers. Based on the apparent intraspatial organization of regions of high activity, this worker proposed a specific secretory apparatus and further suggested the possibility of a subcutaneous pore through which the enzyme could be released to the root surface. Bieleski and Johnson (1) studied the psi induction and location of Apase in duckweed (S. oligorrhiza). They showed that Apase in control plants was located primarily in and around the vascular strands. In Pi-deficient plants, psi Apase activity was 10 to 20 times control with enzyme activity primarily located in the epidermis of the root and undersurface of the frond, the tissue locations most likely to be in contact with the external environment. They placed plants in solutions containing phosphatase substrates. Control plants hydrolyzed the substrates at 4 to 8% of the rates of tissue homogenates whereas Pi-deficient plants hydrolyzed substrate at 30 to 40% of the homogenate rate. When 32P-labeled glucose-1-phosphate was supplied to intact plants, more 32Pi appeared in the medium than in the tissue. The pattern of appearance was consistent with hydrolysis of substrate in the external apoplast followed by uptake into the cell.

A number of other workers have found plant Apase activity in the cell wall secreted by roots into the external medium. Oparka and Johnson (17) found Apase in the intercellular spaces of Nymphaeoides pelata. Hirata et al. (12) observed psi stimulation of excretion of Apase activity into the external medium with both wheat and rice seedlings. Kummerova (13) showed a psi increase in the activity of phosphatases on the root surfaces of Zea mays. This enhancement was especially pronounced in new roots.

As it became clear that phosphate starvation induced large and often tissue-specific enhancement in Apase activity, a number of workers became interested in identifying and characterizing psi Apases in higher plants. Ninomiya et al. (16) resolved the extracellular Apase activity of tobacco cells in suspension culture into three fractions by sequential chromatography. Two of these fractions were neutral pyrophosphatases with diesterase activity, which showed a pH optimum at 6.8. The third fraction showed the psi enhancement in activity. This Apase had a broad substrate specificity with a pH optimum at 5.8.

Reid and Bieleski (21) identified three Apase isozymes in S. oligorrhiza. Pi-sufficient plants exhibited only one isozyme of Apase. Under Pi-deficiency conditions, total Apase activity increased by 50 times and this was accompanied by the appearance of two lower mol wt Apase isozymes. However, the activity of all three isozymes increased under psi conditions. A number of other workers have identified Apase isozymes in higher plants but have not explored the relationship between phosphate deficiency and enzyme activity (20 and references therein).

Data from a number of different experimental systems indicate that plants have evolved starvation rescue systems composed of psi gene systems which effectively act to increase the concentration of exogenous Pi (9, 21, 24).

In 1960, Hewitt and Tatham (11) showed that Apase activity in tomato leaf tissue increased up to 18 times normal under Pi-deficiency conditions. By mixing extracts from Pi-sufficient and Pi-deficient plants, these workers demonstrated that changes in enzyme activity were not due to soluble enzyme regulatory factors. A psi enhancement of Apase activity has also been shown for Euglena gracilis (22), Nicotinia tabacum cell cultures (24-26), Spriodela oligorrhiza (21), Ochromonas dancia (19), Impomoea sp. (28), Triticum aestivum (12), Oriza sativa (12), Zea mays (5, 13), Agrostis capillaris (15), and Arabadopsis thaliana (our unpublished data).

A number of workers have studied the distribution of Apase within plant tissues. De Jong (4) localized Apase in onion roots.

1 Publication No. 4470 of the Arizona Agricultural Experiment Station.
2 Abbreviations: psi, phosphate starvation inducible; Apase, acid phosphatase; Pi, orthophosphate; epsi, excreted phosphate starvation inducible; CAP, crude acetone precipitate.
Excreted Phosphate Starvation Inducible Aspase of Tomato

We have recently reported on the regulation of the ϕ Aspase in suspension cultured cells of tomato and shown that, while excreted Aspase activity increases with phosphate starvation, the intracellular activity remains the same in both ±Pi cells (9, 10). We have further shown that ϕ Aspase enhancement of excreted Aspase occurs before starved cells show any measurable decrease in biomass accumulation. These data suggest a specific ϕ Aspase gene regulation and signal sequences and further suggest that plants will have other ϕ genes that may be regulated in a coordinated manner as a pho regulon (23).

In this article, we characterize the ϕ Aspase from L. esculentum. We further show, for the first time, the complex pattern of changes in steady state levels of excreted proteins under ϕ conditions in higher plants. Induction of the ϕ response occurs within 24 h of transfer to the Pi-depleted medium, whereas the growth rate of the ±Pi cells remains the same as the +Pi cells for up to 8 d (9). These data support the concept of a biochemical early warning system for the sensing of, and response to, phosphate depletion in the external medium.

Materials and Methods

All experiments were carried out with Lycopersicon esculentum (cv VF36). Plant cell suspension culture methods and quantitative assays of Aspase were done as described in the preceding paper (10).

The ϕ Aspase was isolated from the supernatant of suspension cultures and chromatographed on Sephadex G-150 following the methods of Ninomiya et al. (16). Supernatant from 3-d-old cells was gravity filtered through 150 μm nylon mesh. At this time, the ±Pi cells excreted 2.7 times the ϕ Aspase activity of the +Pi treatment (Fig. 1a of Ref. 10). The filtrate was centrifuged at 23,000g at 4°C for 15 min. The supernatant was decanted and mixed 1:1 with ice-cold acetone and allowed to precipitate overnight. The precipitate was pelleted at 3,300g at 4°C and vacuum-dried for 8 h. The pellet was resuspended gently (Polytron, speed 3, 5 s) in 0.01 M Na acetate, 1.0 M NaCl (pH 5.6) (one-tenth volume of the original supernatant). The mixture was stirred overnight at 4°C and insoluble material repelleted at 180,000g at 4°C for 4 h. Five ml of the supernatant was applied to the G-150 column (1.5 × 70 cm) and eluted at 6 ml/h with 0.01 M Na acetate, 0.5 M NaCl (pH 6.0). Aspase activity was resolved into two fractions (Fig. 1). The apparent high mol wt peak has been designated M I by Ninomiya et al. (16) while the apparent low mol wt peak was designated M II.

Enzyme activity was assayed using p-nitrophenyl phosphate (Sigma), and 40 mg of β-naphthyl acid phosphate (Sigma, Fig. 2) at room temperature in the dark.

M I and M II were assayed for the effect of pH on enzyme activity (Fig. 3). The assay conditions were as described above except for the substitution of 0.1 M buffering agents adjusted to their pKs. Equal volumes of enzyme were added and relative activities were determined at pH 3.8 (formate), 4.7 (citrate), 5.6 (succinate), 6.1 (Mes), 7.5 (Tes), and 8.1 (Tris). No change in pH was observed during any of the assays.

SDS-PAGE was carried out on CAP, M I, and M II (Fig. 4). The gel and buffer system were after Laemmli (14), with the exception that the samples were mixed with 5% SDS only and loaded onto the gel. Electrophoresis was carried out at 4°C. The gel was rinsed for 1 h at room temperature in 0.25 M Na acetate (pH 5.6), and activity stained as described above. For mol wt determinations, protein standards were run alongside the Aspase samples. These standards were in 2% SDS, 60 mM DTT, 15% sucrose, 5 mM amino-N-capric acid, and 1 mM benzamidene and were boiled for 3 min before loading. Sequential staining for Aspase activity followed by Coomassie blue protein staining allowed for the visualization of both Aspase activity and mol wt standards.

[35S]Methionine-labeling of proteins excreted by suspension cultured tomato cells was conducted as follows. A 1.5 ml aliquot was removed from 3-d-old ±Pi cells and placed in a sterile, shallow-bottomed, 2 ml microcentrifuge tube. Forty-four μCi of [35S]methionine (11 μCi/μl) were added, and the tubes were taped flat to a gyratory shaker and incubated at 300 rpm for 8 h. Two ml of the supernatant was applied to the G-150 column (1.5 × 70 cm) and eluted at 6 ml/h with 0.1 M Na acetate, 0.5 M NaCl (pH 6.0). Aspase activity was resolved into two fractions (Fig. 1). The apparent high mol wt peak has been designated M I by Ninomiya et al. (16) while the apparent low mol wt peak was designated M II.

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Results

Figure 1 shows the elution profile of Aspase activity from the G-150 column for 3-d-old cells ±Pi. As previously shown (Fig. 1a of Ref. 10), significant differences in ϕ Aspase activity were seen at 24 h, with peak induction occurring in the ±Pi cells at 9 d. In contrast, there was no significant difference in the biomass accumulation rates of the two cultures for the first 8 d. At 3 d, the ±Pi cells had an average of 2.7 times the excreted Aspase activity of the +Pi treatment. Assay of total ϕ Aspase activity in the extracellular supernatants of the specific cultures used for these chromatograms showed a threefold enhancement in en-

![Graph](https://via.placeholder.com/150)

FIG. 1. Sephadex G-150 elution profile of Aspase activity in the concentrated supernatant of suspension cultured cells grown +Pi (O) or −Pi (Δ). Activity is normalized per g of fresh weight of cells.
zyme activity for the −Pi culture. Integration of the curves in Figure 1 shows that −Pi M I (high mol wt) had 1.3-times the Apase activity of +Pi M I while M II (low mol wt) had 2.4-times enhancement. Integration of the entire chromatogram gave 2.3-times the total Apase activity in the −Pi sample.

Figure 2 shows the results of activity staining of native PAGE assays of CAP, M I, and M II. M I showed a large, poorly defined region of activity near the top of the running gel. M II resolved into two bands of activity in the middle part of the gel. CAP showed a small amount of M I activity and the two M II bands. The behavior of M I and M II on the gel generally reflected their behavior on the G-150 column.

As shown in Figure 3, the pH versus activity profiles of M I and M II differed. Both enzymes displayed high levels of activity in the low pH range. The activity of M II dropped off quickly above pH 6.5, showing expected acid phosphatase behavior. M I exhibited high levels of activity even at pH 8.1 which functionally defines it as both an acid and an alkaline phosphatase. This pH versus activity profile becomes even more interesting in view of the data presented in Figure 4. Treatment with SDS resolved both M I and M II to a single band of enzyme activity with an apparent mol wt of 57 kD. Boiling the samples for 2 min (in SDS only) substantially decreased activity staining but did not

![Figure 2](image1)

**Fig. 2.** Apase activity stain of native PAGE of CAP, M I, and M II.

![Figure 3](image2)

**Fig. 3.** Apase activity versus pH profiles of M I (○) and M II (△).

![Figure 4](image3)

**Fig. 4.** SDS-PAGE followed by activity staining of CAP, M I, and M II.

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**Table 1. Effect of Desalting/Resalting on the Apparent Mol Wt of the M II Fraction of the epi Apase**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>M II in buffer (0.558)</th>
<th>Diaflow, 50 kD filter</th>
<th>filtrate</th>
<th>(0.006)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>M II in buffer (0.558)</td>
<td>Diaflow, 50 kD filter</td>
<td>filtrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>M II in buffer (0.558)</td>
<td>Diaflow, 50 kD filter</td>
<td>retentate in buffer</td>
<td>(0.551)</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>M II in buffer (0.558)</td>
<td>Diaflow, 50 kD filter</td>
<td>retentate in water</td>
<td>(0.538)</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Treatment 2</td>
<td>Diaflow 300 kD</td>
<td>filtrate</td>
<td>(0.529)</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Treatment 3</td>
<td>Diaflow 300 kD</td>
<td>filtrate</td>
<td>(0.055)</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Treatment 2</td>
<td>Diaflow 300 kD</td>
<td>retentate in buffer</td>
<td>(0.049)</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Treatment 3</td>
<td>Diaflow 300 kD</td>
<td>retentate in buffer</td>
<td>(0.517)</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Treatment 7</td>
<td>Diaflow 300 kD</td>
<td>filtrate</td>
<td>(0.581)</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Treatment 7</td>
<td>Diaflow 300 kD</td>
<td>retentate in buffer</td>
<td>(0.027)</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Treatment 8</td>
<td>Diaflow 50 kD</td>
<td>filtrate</td>
<td>(0.025)</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Treatment 8</td>
<td>Diaflow 50 kD</td>
<td>retentate in buffer</td>
<td>(0.595)</td>
<td></td>
</tr>
</tbody>
</table>

The treatments are given sequentially from left to right. The absorbance unit values in parenthesis are at 400 nm and represent the result of NPP assays of enzyme activity in the same volume as the original sample. Buffer = 0.01 m Na acetate, 0.5 m NaCl (pH 6.0).

significantly alter the position of the band (not shown). The data shown in Table I also suggest that this enzyme can form high mol wt aggregates in a reversible manner. In these experiments, the M II fraction from the Sephadex G-150 column was pressure-filtered through a 50 kD filter, resuspended in either buffer or water, and passed through a 300 kD filter. Resuspension of the M II retentate in water resulted in almost all enzyme activity being retained on the 300 kD filter, whereas resuspension in
buffer resulted in almost all activity passing through the 300 kD filter. This effect was completely reversible and could be repeated several times on the same sample.

Figure 5 shows the differences in the steady state level of [35S]methionine labeling of excreted proteins from ±Pi cells. Several new proteins are synthesized and at least one is repressed under psi conditions. In addition, there is an approximate twofold increase in the amount of two labeled proteins in the 60 to 70 kD size range.

**DISCUSSION**

The data presented here clearly demonstrate the psi nature of an excreted acid phosphatase in tomato. We propose that enzymes in this category be called epsi Apases. In addition, examination of a population of excreted proteins from suspension-cultured cells shows that the psi response involves both changes in the steady state level of existing proteins and apparent de novo protein synthesis. As shown in Figure 1 of the preceding paper (10), the psi response is observed long before there is any change in cellular growth rate as measured by biomass accumulation.

These data strongly suggest that the types of sensing and regulatory components known to exist in both the *E. coli* (23) and *S. cerevisiae* (27) pho regulons are present in plants as well. Beli and Johnson (1) discussed the evidence that intracellular Pi is maintained in two separate compartments, one of which (presumably the tonoplast) exchanges only slowly with the second labile (presumably cytosolic) metabolic Pi pool. This pool is rapidly depleted when the exogenous Pi is removed. Therefore, cellular growth processes will suffer apparent Pi starvation before total intracellular Pi is depleted.

The approximate threefold enhancement in epsi Apase activity seen in 3-d-old cells of ±Pi was retained through Sephadex G-150 chromatography. Using the terminology of Ninomiya et al. (16), the faster eluting (high mol wt) peak of activity is M I while the slower eluting (lower mol wt) peak is designated M II. Using tobacco suspension cultures, these workers also observed that the majority of the psi enhancement occurred in the M II fraction. Integration of the chromatograms in Figure 1 showed that the M I activity in the −Pi fraction is 1.3 times that of the +Pi. M II activity in the −Pi was 2.4 times that of the +Pi. By use of the small contribution of M I, total enhancement of activity was 2.3 times. This was in reasonable agreement with the enhancement seen in the culture supernatant (Fig. 1 of Ref. 10). A similar ratio was observed by Ninomiya et al. (16).

These data provide an initial characterization of the epsi Apase of tomato. We propose that the excreted Apase in tomato is phosphate starvation inducible and either has two isozymes or is composed of two subunits that retain Apase activity when separated by native PAGE. Under certain conditions, this enzyme is capable of aggregation into high mol wt units that not only retain Apase activity but also exhibit significant alkaline phosphatase activity not shown by the lower mol wt unit.

The epsi Apase from tobacco characterized by Ninomaya et al. (16) was similar to that of tomato. Their M II hydrolyzed a broad range of substrates and had an apparent mol wt of 90 kD by gel permeation chromatography. As discussed by Paul and Williamson (20), tomato contains multiple Apase isozymes. These workers characterized Apase-I from *L. esculentum*. At the present time, it is not clear which, if any, of the previously characterized Apases corresponds to the epsi Apase. We note that the epsi response is similar in tomato lines that have not had the nematode resistance gene and its associated Apase-I gene introduced from *L. peruvianum* (data not shown).

Depletion of Pi from the medium has immediate and dramatic effects on both the types and steady state levels of proteins excreted from the cell (Fig. 5). Two protein bands are present in the −Pi supernatants that either are not present or are present at a very low level in the +Pi treatment. In addition, the steady state levels of several bands were enhanced in the −Pi supernatant, including a twofold increase (as measured by scanning laser densitometer, not shown) in the labeling of two bands at approximately 60 to 70 kD (uppermost arrow, Fig. 5). The increase in band intensity in this mol wt range corresponds approximately with the observed 2.7-fold increase in Apase activity. The 57 kD mol wt value was obtained using protein samples treated only with 5% SDS so that Apase activity could be assayed. The samples for the autoradiograms were boiled for 3 min with SDS and β-mercaptoethanol (14). One or both of these differences in the protocol might account for the slightly higher apparent mol wt observed on the autoradiogram. Alternatively, it is possible that neither of the bands in the 60 to 70 kD range is the epsi Apase since we have determined that this protein is only approximately 4% of total soluble excreted protein at late log phase and would therefore be less than that at 3 d (data not shown). It is important to reemphasize that these changes were observed far
in advance of any change in the relative growth rate of the –Pi cells with respect to the +Pi cells.

The data presented here, when viewed in conjunction with the previous work by ourselves and others, strongly suggest the existence of a multigene phosphate starvation rescue system in higher plants. As a working hypothesis, it is reasonable to propose that at least some of these genes share a common trans-acting factor. This set of genes would form the pho regulon (23). The need for a pho regulon in higher plants is easily justified because the level of Pi in the solution phase of soils is often below those of many micronutrients (6). In soils, all major nutrient ions except Pi are normally present at concentrations from 1.0 to 1.0 mM whereas the Pi concentration is commonly 1.0 μM or less. Ozanne (18) has concluded that, in many natural ecosystems, Pi is the growth-limiting element due to its low concentration in the soil solution. In contrast, the soil often contains large amounts of both organic and insoluble mineral P (7).

The current picture of psi metabolism is complex and involves integrated cellular, tissue, and whole plant responses. At the present time, the existence of a higher plant pho regulon may be inferred from anatomical, physiological, and biochemical events triggered by phosphate starvation. These events include tissue specific synthesis of an apoplastic Apease, enhanced root growth, and increased rates of Pi uptake and bidirectional transport (2, 3). It is probable that further study of psi metabolism will add to our understanding of the regulation of plant gene expression. In addition, organic and mineral phosphate solubilizing genes (7, 8) could play a role in the development of biological P fertilizers.

LITERATURE CITED

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