CHAPTER II

SECTION A

SEPARATION AND PARTIAL PURIFICATION OF ACID PHOSPHATASES FROM THE COTYLEDONS OF 48-h GERMINATING SEEDS OF V. sinensis
INTRODUCTION

Multiple forms of acid phosphatase have been identified in a large number of sources, e.g. rabbit, lymphoid cell, frog liver, drosophila, red cell, human prostate and placenta, various plants and microorganisms. In some cases specific phosphatases have been found in plasma membranes or lysozomal membranes or loosely bound to a cell surface. Characterization of these enzymes has usually revealed little substrate specificity. Assessment of the functional significance of acid phosphatase has been based on their occurrence in lysosomes, their elevated specific activities in necrotic tissues, and the histochemical demonstration of phosphatase activity in the multitude of tissues under various physiological conditions. Because of this nonspecificity and ubiquity, the functional significance of acid phosphatases has remained largely obscure.

Several attempts have been made in the last few years in the isolation, purification and characterization of the enzymes of phosphate metabolism, mainly phosphatase, pyrophosphatase and phytase. Results presented in the earlier chapter indicate that there is extensive variation in the expression of acid phosphatase activities with the time of germination. Consequently, such variation raises questions regarding the biological functions as well as the structural and evolutionary relationship between the multiple forms of acid phosphatase. At least six forms of acid phosphatase in the cotyledon of 48-h germinating seeds of V. sinensis...
were well established on the basis of gel electrophoretic pattern (165). The separation and purification of these isozymes should permit a detailed analysis of the physico-chemical differences between themselves.

This section is mainly concerned with the isolation and purification of four different forms of acid phosphatase present in the germinating seeds of V. sinensis. These isozymes were separated with the help of usual protein purification techniques *viz.* ammonium sulfate fractionation, heat denaturation, ion exchange chromatography and gel filtration techniques.

**MATERIALS**

Sephadex G-100, Sephadex-200 were the products of Pharmacia Fine Chemicals (Sweden). DE-52 was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ammonium sulfate, Triton X-100 and polyethylene glycol 6000 were purchased from BDH, England. Tris was also from Sigma Chemical Co.

**METHODS**

**Germination of Seeds:**

Seeds of *Vigna sinensis* were washed in tap water and immersed in 0.02% HgCl₂ for 10 min (for surface sterilization) followed by several changes with distilled water. Seeds were decoted and allowed to germinate at dark at 28°C.
Preparation of Cell-free Extract:

Cotyledons of 48-h germinating seeds of *V. sinensis* were separated from the embryo and kept in ice cold condition. After washing with distilled water, a 10% homogenate of the cotyledon was made with ice cold buffer (10 mM Tris-HCl, pH 7.4) containing 0.1% Triton X-100, by grinding with sand in a mortar and pestle. The homogenate was kept in cold condition overnight and was strained through double layer of cheesecloth to remove coarser cell debris. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C in a refrigerated centrifuge (Model B-20) to remove the remaining cell debris. The supernatant thus obtained was further centrifuged at 10,000 x g for 20 min at 4°C. The pellet was discarded and the supernatant was used as the starting material for purification of acid phosphatases.

Heat treatment at 50°C:

The 10,000 x g supernatant was heated at 50°C for 10 min and then placed in ice bath for 30 min. The denatured protein was then discarded after centrifugation at 10,000 x g for 20 min and the supernatant was saved for further purification. All subsequent operations were carried out at 4° - 8°C.

Ammonium sulfate precipitation:

Solid ammonium sulfate (11.8 g/100 ml of supernatant) was added slowly with constant stirring. The mixture was stirred for additional 60 min and kept overnight. The suspension was then centrifuged at 10,000 x g for 20 min to remove the precipitated...
protein (30% ammonium sulfate fraction). The supernatant was then further fractionated by adding 35.6 g of ammonium sulfate/100 ml of supernatant and treated as above (30-80% ammonium sulfate fraction). The precipitate thus obtained (30-80% ammonium sulfate fraction) was suspended in 60% ammonium sulfate solution and stirred overnight. This suspension was then centrifuged at 10,000 x g for 20 min and the supernatant was discarded. The pellet was dissolved in 10 mM Tris-HCl buffer (pH 7.4). This fraction was designated as 30-60% ammonium sulfate fraction and subsequently dialysed against the same buffer (5 litres) for 12 h. After dialysis, this fraction was centrifuged to remove the undissolved materials and denatured proteins.

**DE-52 Column Chromatography:**

Dry material (DE-52) was suspended in 1 N NaOH and washed with distilled water to make it free from excess alkali. 1 N HCl was added sufficiently to make a strong suspension, filtered immediately, washed with distilled water to remove the free acid. This activated form of DE-52 was suspended in 10 mM Tris-HCl buffer (pH 7.4). The matrix was poured into a column (36 x 2.4 cm) to make it ready for enzyme purification. The DE-52 column was equilibrated with 10 mM Tris-HCl buffer (pH 7.4) until the pH of the effluent was identical with that of the applied buffer. After equilibration of the DE-52 column, the dialysed 30-60% ammonium sulfate containing 300 mg of protein was charged onto it. The column was then washed with 500 ml of 10 mM Tris-HCl buffer (pH 7.4) followed by 0.05 M KCl in the same buffer. Elution was carried out with 1,000 ml of KCl solution in the same buffer with
a linear gradient of 0.05 M to 0.3 M. The flow rate of the column was adjusted to 30 ml/h. Fractions containing 10 ml each were then collected using an automatic fraction collector (Buchler Fractometer 200). The enzyme activity was assayed with each fraction and the zymogram pattern of acid phosphatase from the active fractions was also studied by polyacrylamide gel. The elution profile has been shown in Fig. 22. Active fractions were pooled in four classes according to the presence of different isozymes of acid phosphatase: Fraction I (Fr. 17-19), Fraction II (Fr. 20-26), Fraction III (Fr. 27-60) and Fraction IV (Fr. 61-65). Each of the pooled fractions was dialysed against 5 litres of 10 mM Tris-HCl buffer (pH 7.4) for 12 h with several changes to remove the salt. After exhaustive dialysis the fraction was dialysed against polyethylene glycol (PEG) to concentrate the protein and then with 10 mM Tris-HCl buffer (pH 7.4) to remove PEG.

Gel Filtration:

Sephadex G-100 and Sephadex G-200 gel, which were used for the purification of acid phosphatase, were allowed to swell in excess water by putting the gel on a boiling water bath for 5 h. The column was then mounted vertically and the dead space under the net in the tubing filled with 10 mM Tris-HCl buffer (pH 7.4) and this was done by injecting the eluent (10 mM Tris-HCl buffer, pH 7.4) into the outlet tubing and pumping it up through the bed subordinate until the dead space was properly filled. The rest part of the column was filled with the same eluent. The swelled
The concentrated pooled Fraction I from DE-52 chromatography was loaded onto the Sephadex G-100 column (112 x 1.4 cm), which was previously equilibrated with 10 mM Tris-HCl buffer (pH 7.4). 2 ml fractions were then collected using the automatic fraction collector, at a flow rate of 6 ml/h. The elution profile has been shown in Fig. 24.

Gel Filtration of pooled fraction II from DE-52 through Sephadex G-200:

A Sephadex G-200 column (75 x 1 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 7.4). The concentrated pooled Fraction II from DE-52 was charged onto the previously equilibrated Sephadex G-200. Elution was performed with the same buffer at a flow rate of 6 ml/h and 1.5 ml fractions were collected using the automatic fraction collector. The elution profile has been shown in Fig. 26.

The active fractions were pooled in three parts according to the presence of different acid phosphatase isozymes and designated as pooled Fraction I’ (Fr. 11-14), pooled Fraction II’ (Fr. 15-22) and pooled Fraction III’ (Fr. 23-28).
Ge3> Filtration of pooled Fraction I' and III' from Sephadex G-200 gel and pooled Fraction IV from DE-52 chromatography through Sephadex G-100:

A Sephadex G-100 column (112 x 1 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at a flow of 6 ml/h. The pooled Fraction I' obtained from Sephadex G-200 column was concentrated against polyethylene glycol 6,000 and dialysed against the 10 mM Tris-HCl buffer (pH 7.4) to remove the PEG. The concentrated materials were applied to the previously equilibrated gel and the elution was performed with the same buffer at the same flow rate (Fig. 27). This Sephadex G-100 column was made ready for another gel filtration. The pooled Fraction III' was similarly charged onto the column and the whole operation was carried out like the previous one (Fig. 28). The active fractions were pooled by studying the zymogram pattern of acid phosphatase into three parts and designated as pooled Fraction I'' (Fr. 16-21), pooled Fraction II'' (Fr. 22-26) and pooled Fraction III'' (Fr. 27-30).

Sephadex G-100 gel filtration of concentrated pooled fraction IV from DE-52 chromatography was similarly operated as before (Fig. 29).

Preparative polyacrylamide gel electrophoresis:

The preparative polyacrylamide gel electrophoresis was performed according to the method of Davis (169). Histidine buffer (pH 8.5) was used as gel buffer and 9.0 cm gel was prepared (monomer concentration was 7.0%). The electrophoresis was carried out at 3 mA/Tube. After electrophoresis, the activity of acid
phosphatase was visualized in the polyacrylamide gel by the diazo-dye method described by Brewer (170). The composition of the staining mixture was described in the "Methods" of Section II in Chapter I.

**Enzyme Assay:**

The assay mixture, in a total volume of 2 ml, contained 0.5 ml of 0.1 M sodium acetate buffer (pH 5.0), 0.05 ml of 0.025 M p-nitrophenyl phosphate and required amount of enzyme source. Incubation was carried out at 37°C for the period as indicated in the Figure legend. The reaction was stopped by the addition of 4 ml of 0.1 N NaOH. The color thus developed was estimated at 410 μm in a Bausch and Lomb (Spectronic 20) (112).

**Estimation of Protein:**

Protein was determined by the method of Lowry et al. (115) or by measuring absorbance at 205 μm.
Tables 8 and 9 represent the summary of the purification procedure. AP-I enzyme has been purified 75.8 fold, AP-II to 38.4 fold, AP-IV to 16 fold and AP-V to 38.7 fold over the crude enzyme preparation from the 48-h germinating seeds of *V. sinensis*. Schematic representation of the separation of the four forms of acid phosphatase has also been shown in Fig. 20.

The crude extract (16,000 x g) was heated for 10 min at 50°C with the loss of 10% of the total activity. The zymogram pattern showed that there was practically no change of the acid phosphatase activity after the heat treatment. The heat labile proteins were eliminated in this step. 30-60% Ammonium sulfate fraction contained most of the isozymes of acid phosphatase present in the 48-h germinating seeds of *V. sinensis* (Fig. 21). This fraction was taken as the enzyme source for further purification. An aliquot of the 30-60% ammonium sulfate fraction containing 300 mg protein was charged onto a DE-52 column and the protein was eluted by KCl (50 mM to 300 mM) with the broad elution profile (Fig. 22). The zymogram pattern of acid phosphatases in different fractions showed that only AP-I was eluted at the early fractions (Fr. 16-19), but AP-I, AP-II and AP-IV came out in the fractions from 20 to 26. However, all the phosphatases were eluted mainly in 27-60 fractions. The AP-V predominately came out in the fractions 61-65. Figure 23 represents the electrophoretic mobility of different acid phosphatases present in the different fractions of DE-52 column. According to the distribution pattern of isozymes of acid phosphatase...
in the different fractions (as revealed by zymogram technique), they were pooled separately e.g. pooled Fraction I, pooled Fraction II, pooled Fraction III and pooled Fraction IV. The enzyme pooled Fraction I (AP-I rich fraction) was further purified by Sephadex G-100 (Fig. 24). Zymogram pattern showed that different fractions contained only acid phosphatase I (Fig. 25). Pooled Fraction II from DE-52 (mainly present AP-II and AP-IV) was charged onto a Sephadex G-200 column and elution was carried with 10 mM Tris-HCl buffer (pH 7.4). The electrophoretic behaviour in the polyacrylamide gel showed that AP-II was mainly eluted in the early fraction but AP-IV was eluted in the later part with a small contamination of AP-I and AP-II (Fig. 26). According to their presence in the different fractions they were pooled separately and designated as pooled Fraction I', pooled Fraction II' and pooled Fraction III'. Pooled Fraction I' from Sephadex G-200 (mainly present AP-II) was further purified by Sephadex G-100 gel filtration (Fig. 27). The polyacrylamide gel electrophoretic pattern showed that AP-I (which was present in small amount in the charging sample) came out only in the middle fractions (Fr. 10-12). Pooled Fraction III' from Sephadex G-200 was also purified by Sephadex G-100. Zymogram pattern showed that AP-I and AP-II (which were present in minute amount in the loading sample) came out in the early fractions (Fr. 16-26), AP-IV eluted in the later part (Fr. 27-30). They were pooled separately and designated as pooled Fraction I", pooled Fraction II" and pooled Fraction III". The pooled Fraction III" contained AP-IV and devoid of any other phosphatases (Fig. 28).

The pooled Fraction IV from DE-52 contained mainly AP-II and AP-V which were further separated by Sephadex G-100 gel
filtration. Zymogram pattern of all the fractions from Sephadex G-100 was determined. The results presented in Fig. 29 suggest that AP-II isozyme was present up to fraction 39. In the rest of the fractions (Fr. 40-60) only AP-V was found to be present.

Fig. 30 showed the relative mobility of four partially purified acid phosphatase in 7% acrylamide gel electrophoresis.
Table 8

Purification chart of acid phosphatase from 48-h germinating seeds of V. sinensis

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (activity/mg)</th>
<th>Fold of purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 x g</td>
<td>5,000</td>
<td>31,360</td>
<td>6.25</td>
<td></td>
</tr>
<tr>
<td>Heated fraction at 50°C for 10 min</td>
<td>2,203</td>
<td>27,940</td>
<td>12.7</td>
<td>2.0</td>
</tr>
<tr>
<td>30-60% ammonium sulfate fraction</td>
<td>904</td>
<td>22,600</td>
<td>25.0</td>
<td>4.0</td>
</tr>
<tr>
<td>DE-52 pooled I</td>
<td>6.0</td>
<td>780</td>
<td>130.0</td>
<td>20.8</td>
</tr>
<tr>
<td>&quot; pooled II</td>
<td>56.0</td>
<td>2,400</td>
<td>42.9</td>
<td>6.9</td>
</tr>
<tr>
<td>&quot; pooled III</td>
<td>170.2</td>
<td>4,000</td>
<td>23.5</td>
<td>3.8</td>
</tr>
<tr>
<td>&quot; pooled IV</td>
<td>7.0</td>
<td>350</td>
<td>50.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>
Table 9
Specific activities of four different isozymes of acid phosphatase, after final purification

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (activity/mg protein)</th>
<th>Fold of purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase I (AP-I)</td>
<td>0.28</td>
<td>132</td>
<td>468.8</td>
<td>75.9</td>
</tr>
<tr>
<td>Acid phosphatase II (AP-II)</td>
<td>0.58</td>
<td>138</td>
<td>238.0</td>
<td>38.4</td>
</tr>
<tr>
<td>Acid phosphatase IV (AP-IV)</td>
<td>0.21</td>
<td>210</td>
<td>100.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Acid phosphatase V (AP-V)</td>
<td>1.10</td>
<td>266</td>
<td>242.0</td>
<td>38.7</td>
</tr>
</tbody>
</table>
Schematic representation on separation of four different isoenzymes of acid phosphatase from germinating seeds of Vigna sinensis.

Cell-free extract of 8-h-old cotyledons
Heated at 50°C for 10 min
Centrifuged at 15,000 x g for 10 min.

Supernatant
\((\text{NH}_4)_2\text{SO}_4\) Fractionation
30–60% Ftr.
DE-52 Column Chromatography,
Gradient elution with 50–300 mM KCl in
10 mM Tris-Cl buffer.

Pool Fr. I
17–19
Pool Fr. II
20–26
Pool Fr. III
27–60
Pool Fr. IV
61–65

Separation of AP-I

DE-52 Fr. I
Gel filtration through Sephadex G-100
(Fig. 24)
Purified AP-I

Separation of AP-II & AP-IV

Pool Fration II
From DE-52
Gel filtration through Sephadex G-200
(Fig. 26)
Pool Fration II
11–14
Pool Fration II
15–22
Pool Fration III
23–28

Separation of AP-V

Pool Fration IV
From DE-52
Gel filtration through Sephadex G-100
Purified AP-V
(Fig. 22)
Pool Fration IV
AO-60

Purified Fraction

Gel filtration through Sephadex G-100
(Fig. 27)
Purified AP-II
Pool Fration I
16–21
Pool Fration II
22–26
Purified AP-IV
Pool Fration III
27–30
Fig. 20: Schematic representation of the separation of four different isozymes of acid phosphatase from the cell-free extract of germinating seeds of V. sinensis.

Fig. 21: Polyacrylamide gel electrophoretic pattern of acid phosphatase activity present in various ammonium sulfate fractions. Gel electrophoresis and staining procedures were described in 'Methods' of this section. Tube 1, materials from crude enzyme system; Tube 2, materials from 30-60\% ammonium sulfate fraction; Tube 3, materials from 60-80\% ammonium sulfate fraction.

Fig. 22: Chromatography of acid phosphatase (30-60\% ammonium sulfate fraction) on a DE-52 column (36 x 2.4 cm). The sample containing 300 mg of protein was applied to the column previously equilibrated with 10 mM Tris-HCl buffer (pH 7.4). The column was washed with 10 mM Tris-HCl buffer (pH 7.4) followed by 50 mM KCl in the same buffer. The effluents were discarded. The column was finally eluted with a linear gradient between 500 ml of 50 mM KCl and 500 ml of 300 mM KCl in 10 mM Tris-HCl buffer (pH 7.4) at a flow rate of 30 ml/h. Fractions of 10 ml were collected and assayed for acid phosphatase activity as described in 'Methods' of this section. The enzymatic activity of each fraction was plotted against the fraction number.
Polyacrylamide gel electrophoretic pattern of acid active phosphatase activity present in the fractions eluted from DE-52 column chromatography. Each of the active fractions was dialysed separately against 10 mM Tris-HCl buffer (pH 7.4) to remove the salt, against polyethylene glycol 6,000 to concentrate the protein and against the same buffer to remove the polyethylene glycol. After mixing with glycerol (20% final) the concentrated sample (100 μl) was loaded on each of the polyacrylamide gels, electrophoresed and stained as described in 'Methods' of this section. Tube 1, materials from 30-60% ammonium sulfate fraction; Tube 2, materials from fraction No. 16 from DE-52 chromatography; Tube 3, materials from fraction No. 20 from DE-52 chromatography; Tube 4, materials from fraction No. 22 from DE-52 chromatography; Tube 5, materials from fraction No. 29 from DE-52 chromatography; Tube 6, materials from fraction No. 46 from DE-52 chromatography; Tube 7, materials from fraction No. 62 of DE-52 chromatography.

Sephadex G-100 gel filtration of pooled Fraction I from DE-52 chromatography. Pooled Fraction I (Fr. 17-19) from DE-52 chromatography (described in Fig. 22) was dialysed against 10 mM Tris-HCl buffer (pH 7.4) to remove the salt, against polyethylene glycol to concentrate the protein and against the same buffer to remove polyethylene glycol. This concentrated fraction
containing 2.0 mg of protein was applied to a Sephadex G-100 column (112 x 1.0 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Elution was performed with the same buffer at a flow rate of 6 ml/h and 2.5 ml fractions were collected. The acid phosphatase activity in each fraction was assayed and plotted against the fraction number.

Fig. 25: Polyacrylamide gel electrophoretic pattern of acid phosphatase activity present in the eluted fractions from Sephadex G-100 gel filtration. Different active fractions were dialysed against polyethylene glycol 6,000 to concentrate the protein and against 10 mM Tris-HCl buffer (pH 7.4) to remove polyethylene glycol. The concentrated samples (100 μl) were mixed with glycerol (20% final) and were loaded on each of the polyacrylamide gels. Electrophoresis and activity staining were carried out as described in 'Methods' of this section. Tube 1, 30-60% (NH₄)₂SO₄ fraction; Tube 2, pooled Fraction I from DE-52; Tube 3, 4, 5, 6, 7, 8, 9, fraction no. 37, 40, 43, 46, 49, 51 respectively of Sephadex G-100 gel filtration.

Fig. 26: Sephadex G-200 gel filtration of pooled Fraction II from DE-52 chromatography. Pooled Fraction II (Fr. 20-26) of DE-52 chromatography (described in Fig. 22) was dialysed against 10 mM Tris-HCl (pH 7.4) to remove the salt, against polyethylene glycol 6,000 to concentrate the protein and against the same buffer to remove the polyethylene glycol. The concentrated fraction containing 56.0 mg of protein was applied to a Sephadex G-200 column (75 x 1.1 cm) equilibrated with 10 mM Tris-HCl buffer.
Elution was performed with same buffer at a flow rate of 6 ml/h and 1.5 ml fractions were collected. The acid phosphatase activity in each fraction was assayed and plotted against fraction number.

**Fig. 27**: Sephadex G-100 gel filtration of pooled fraction I' from Sephadex G-200 gel. Pooled fraction I' (Fr. 11-14) from Sephadex G-200 gel filtration (described in Fig. 26) was dialysed against polyethylene glycol 6,000 to concentrate the protein and against 10 mM Tris-HCl buffer (pH 7.4) to remove polyethylene glycol. The concentrated materials containing 3.0 mg of protein was applied to a Sephadex G-100 column (112 x 1 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Elution was performed with the same buffer at a flow rate of 6 ml/h and 1.5 ml fractions collected. The acid phosphatase activity in each fraction was assayed and plotted against the fraction number.

**Fig. 28**: Sephadex G-100 gel filtration of pooled fraction III' from Sephadex G-200. Pooled fraction III' (Fr. 23-28) from Sephadex G-200 gel filtration (described in Fig. 26) was dialysed against polyethylene glycol 6,000 to concentrate the protein and against 10 mM Tris-HCl buffer (pH 7.4) to remove the polyethylene glycol. The concentrated material was applied to a Sephadex G-100 column (112 x 1 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Elution was performed with the same buffer at a flow rate of 6 ml/h and 2.5 ml fractions were collected.
The acid phosphatase activity in each fraction was assayed and plotted fraction number.

Fig. 29: Sephadex G-100 gel filtration of pooled fraction IV from DE-52 chromatography. Pooled fraction IV (Fr. 61-65) from DE-52 chromatography (described in Fig. 22) was dialysed against 10 mM Tris-HCl buffer (pH 7.4) to remove the salt, dialysed against polyethylene glycol 6,000 to concentrate the protein and dialysed against the same buffer to remove polyethylene glycol. The concentrated material containing 2.0 mg of protein was applied to a Sephadex G-100 column (112 x 1 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Elution was performed with the same buffer at a flow rate of 6 ml/h and 1 ml fractions were collected and plotted against fraction number.

Fig. 30: Polyacrylamide gel electrophoretic pattern of four partially purified acid phosphatases. Each purified acid phosphatase was concentrated against polyethylene glycol 6,000 to concentrate the enzyme protein and dialysed against 10 mM Tris-HCl buffer (pH 7.4) to remove PEG. The concentrated enzyme fraction was mixed with glycerol (20%, final) and was loaded on each of the polyacrylamide gels. Electrophoresis and staining were carried out as described in 'Methods' of this section. Tube 1, materials from 30-60% ammonium sulfate fraction; Tube 2, materials from AP-I fraction; Tube 3, materials from AP-II fraction; Tube 4, materials from AP-IV fraction; Tube 5, materials from AP-V fraction.
DISCUSSION

From the results obtained in the present investigation it can be observed that most of the acid phosphatases are present in the cotyledon of 48-h germinating seeds of *V. sinensis*. The crude extract of the cotyledon is processed in the usual methods of protein purification. Most of the heat labile holoenzyme proteins are removed by heat treatment of the crude extract. The enzyme proteins are concentrated in the 30-60% ammonium sulfate fraction. This fraction contains most of the acid phosphatases. All acid phosphatases are partially purified and separated from each other by DE-52 column chromatography. Different fractions containing the enzymatic activity are pooled separately according to the existence of different isozymes of acid phosphatase in the eluted fractions. All partially separated acid phosphatases are further purified by Sephadex G-200 and Sephadex G-100 gel filtration. Schematic representation of the overall separation and purification of the four acid phosphatases (AP-I, AP-II, AP-IV and AP-V) has also been shown in Fig. 20. AP-I is purified by DE-52 column chromatography followed by Sephadex G-100. Similarly AP-II and AP-IV are purified by DE-52 column chromatography followed by two subsequent gel filtrations through Sephadex G-200 and Sephadex G-100. AP-V is also purified by DE-52 column chromatography and by gel filtration through Sephadex G-100. Different steps and the extent of purification of acid phosphatases have been shown in Tables 8 and 9. AP-I has been purified 75.8 fold, AP-II to 38.4 fold, AP-IV to 16 fold and AP-V to 38.9 fold over the crude enzyme preparation (acid phosphatase) present in the cotyledon of 48-h
germinating seeds of *V. sinensis*. Zymogram pattern of all the partially purified acid phosphatases (Fig. 30) also confirmed that these are substantially different from each other in their mobility in the polyacrylamide gel electrophoresis. These differences can be explained only if it is assumed that the enzymes differ in their molecular charges and weight. This difference in their molecular make up probably affects the active site of the enzyme leading to a difference in the activity. In order to investigate any such difference in the molecular make up, the characteristic properties of different isozymes are further studied in the next section (Section B) of this chapter.
CHAPTER II

SECTION B

CHARACTERIZATION OF FOUR PARTIALLY PURIFIED ACID PHOSPHATASES
The purification of different isozymes should permit a detailed analysis of the physico-chemical differences between the isozymes. Also, the knowledge of their intracellular localization might allow more evidences to be obtained about the physiological significance of the developmental changes in the different molecular forms of acid phosphatase. In this perspective, the null-activity mutant represents a critical tool to evaluate the role of the acid phosphatase enzymes in the development of plant as indicated by Bell (190). A well-defined biological function of these phosphatases cannot be arrived without the knowledge of substrate specificity, optimum pH, optimum temperature, etc. So to explore the physiological role, expression variation and diverse molecular make-up of the four partially purified phosphatases, attempt has been made to determine their specificity, optimum pH, optimum temperature, heat denaturation, metal ion requirement, inhibitor test and effect of detergent.

**MATERIALS**

p-Nitrophenyl phosphate, D-glucose-6-phosphate, β-D-glycero-phosphate, D-2-phosphoglyceric acid, D-3-phosphoglyceric acid, D-2, 3-diphosphoglyceric acid, phosphoenolpyruvate, pyrophosphate, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate and phytate were purchased from Sigma Chemical Co., St. Louis, Mo. U.S.A. Alloxan, Triton X-100 were purchased from BDH, England. Sodium fluoride, N-ethylmaleimide, sodium dodecyl...
Acid phosphatase activity was measured by the method of Mechel et al. (112). In a total volume of 1 ml, enzyme was incubated with substrate at a final concentration of 0.1 mM in 0.1 M acetate buffer (pH 5.0) at 50°C for 60 min. The reaction was stopped by 0.1 N NaOH and the liberated p-nitrophenol was measured at 410 mp.

Heat Inactivation:

Each of the four partially purified acid phosphatases was diluted five times in 0.1 M acetate buffer (pH 5.0 and 7.0). Bovine serum albumin was added to the enzyme solution at a final concentration of 1 mg/ml. Preincubation was carried out at the indicated temperature given in the legend of Fig. 34. At different time intervals aliquots were removed and assayed for acid phosphatase activity at 50°C.
RESULTS

Time dependent reaction velocity:

Fig. 31 shows that for each of these four partially purified acid phosphatases there was proportionality between the dephosphorylation of p-nitrophenyl phosphate and the time of incubation at 37°C under the standard conditions of assay; a linearity was maintained up to 120 min of incubation.

Optimum pH:

Fig. 32 shows the effect of pH on the activities of four acid phosphatases. The maximum activities of all these phosphatases were found at pH 5.0 in citrate buffer using p-nitrophenyl phosphate as a substrate. In acetate buffer AP-I, AP-IV and V showed their maximum activities at pH 5.0 while AP-II showed its maximum activity at pH 5.5.

Optimum temperature:

Fig. 33 shows the effect of temperature on the activities of the acid phosphatases. The maximum activities of acid phosphatases I and V were found at 55°C while those of phosphatases II and IV were at 60°C and 65°C respectively.

Substrate specificity:

The relative activities of the phosphatases towards the utilization of natural and artificial substances as substrates were measured considering the substrate activity of p-nitrophenyl
phosphate as 100 per cent. From the data given in Table 10 it is evident that all these phosphatases showed different activities with various substrates. The four phosphatases hydrolysed most of the substrates tested at various rates. AP-I showed maximum activity with pyrophosphate. It also hydrolysed PEP, ADP and ATP to a considerable extent. AP-II has the highest activity toward p-nitrophenyl phosphate. It also hydrolysed pyrophosphate, PEP, ADP and ATP to an appreciable extent. But in comparison to AP-I, it hydrolysed 3-PGA more efficiently. These two phosphatases were also active to some extent on G-6-P, β-glycerophosphate and AMP, but not on 2,3-DPG and phytate. However, in contrast to AP-II, no activity could be found in the case of AP-I using 2-PGA as a substrate. AP-IV and AP-V hydrolysed the PEP with maximum efficiency. AP-IV has a higher activity with ADP than with p-nitrophenyl phosphate. The activities of AP-IV and AP-V were greater than those of AP-I and AP-II towards ATP. It has been recorded that AP-IV and AP-V also showed their activities towards 2,3-DPG but AP-I and AP-II were completely inactive towards this substrate. However, AP-IV and AP-V could hydrolyse G-6-P, β-glycerophosphate and 3-PGA to relatively low extents. It should also be noted that in contrast to AP-V, AP-IV showed no activity with AMP, 2-PGA and phytate as substrates.

**Effects of metal ions:**

Effects of various metal ions on the activities of acid phosphatases have been studied with a view to explore the differences in the enzymatic behaviour of the four phosphatases. The metal ions were added to the buffer enzyme mixture prior to the
addition of the substrate. The substrate was added to the incubation mixture after 20 min. The results are given in Table 11. Ca\(^{2+}\), Mn\(^{2+}\) and Mg\(^{2+}\) showed no inhibitory effect on the activity of any of these acid phosphatases but Mn\(^{2+}\) appeared to possess some stimulatory effect (28\%) on AP-II. Fe\(^{3+}\), Hg\(^{2+}\), Mo\(^{6+}\) and Zn\(^{2+}\) were found to inhibit the activities of all the four acid phosphatases, the extent of inhibition being greater with Hg\(^{2+}\). Co\(^{2+}\) has no effect on AP-I and AP-V but has a strong inhibitory effect on AP-II and slight stimulatory effect on AP-IV. Although Pb\(^{2+}\) has no effect on AP-I and AP-II, it has got some inhibitory effect towards AP-IV and AP-V. Cu\(^{2+}\) inhibited the activities of AP-I, AP-IV and AP-V but not the AP-II.

Effects of inhibitors:

Table 12 shows the effects of some well known inhibitors of acid phosphatase on the enzymatic activities of AP-I, -II, -IV and -V. Alloxan has no inhibitory effect on AP-I and AP-II but little effect was found on AP-IV and AP-V. Sodium fluoride inhibited the activities of all the four phosphatases, the effects being more pronounced in the cases of AP-IV and AP-V. NEM has no inhibitory effect on AP-IV and AP-V, but little inhibitory effect on AP-I and AP-II. PCMB inhibited the activities of all the acid phosphatases, but the inhibitory effect was greater in case of AP-IV and AP-V. EDTA has no effect on the activities of four acid phosphatases.
Heat Inactivation:

Fig. 34 shows the nature of the heat inactivation of the four acid phosphatases in acetate buffer (pH 5.0 & 7.0) for different time periods of incubation at 60°C. AP-I retained 46.3% of its initial activity after 60 min of incubation at 60°C in acetate buffer (pH 5.0), while AP-II, AP-IV and AP-V retained 77.5%, 43.5% and 56.0% respectively of their initial activities after similar treatment. But after incubation at pH 7.0 at a temperature of 60°C for 45 min viz. AP-I, AP-II, AP-IV and AP-V could retain 57.7%, 86.5%, 98.0% and 31.7% respectively of their initial activities. Fig. 35 reveals that after 60 min of incubation at 65°C in the same buffer (pH 5.0), these phosphatases retained 42.0%, 36.8%, 25.0% and 12.5% respectively of their initial activities.

Effects of detergents:

Table 13 shows the effects of Triton X-100 and SDS in different concentrations on the activities of four acid phosphatases. AP-I, AP-II and AP-IV showed their maximum activities in the presence of Triton X-100 whereas the activity of AP-V remained almost unaffected. The maximum activities of these three phosphatases were found in presence of 0.5% Triton X-100. SDS drastically inhibited the activities of all the four phosphatases. However, AP-I and AP-V showed little activity in the presence of 0.01% of SDS, but none was found in higher concentrations of the detergent.
Table 10

Substrate Specificities of Acid Phosphatases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Rate of Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP-I</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>100.0</td>
</tr>
<tr>
<td>D-Glucose-6-phosphate</td>
<td>20.0</td>
</tr>
<tr>
<td>β-D-Glycerophosphate</td>
<td>22.0</td>
</tr>
<tr>
<td>D-2-phosphoglyceric acid</td>
<td>0.0</td>
</tr>
<tr>
<td>D-3-phosphoglyceric acid</td>
<td>25.0</td>
</tr>
<tr>
<td>D-2,3-Diphosphoglyceric acid</td>
<td>0.0</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>70.0</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>110.0</td>
</tr>
<tr>
<td>Adenosine Triphosphate</td>
<td>70.0</td>
</tr>
<tr>
<td>Adenosine Diphosphate</td>
<td>55.0</td>
</tr>
<tr>
<td>Adenosine-5'-monophosphate</td>
<td>20.0</td>
</tr>
<tr>
<td>Phytate</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The enzymes were incubated with each substrate at 0.1 mM for 60 min at 50°C in 0.1 M Na-acetate buffer (pH 5.0). The reaction was terminated by the addition of trichloric acid to a final concentration of 5%. The amount of released free phosphate was measured by the method of Lowry and Lopez (113). All values were expressed as the per cent of the amount of free phosphate released from p-nitrophenyl phosphate.
The enzymes were incubated with substrate and metal ions at the final concentration of 0.1 mM and 1.0 mM respectively for 60 min at 50°C in 0.1 M acetate buffer (pH 5.0). The metal ions were added to the buffer-enzyme mixture 20 min before adding the substrate. The reaction was stopped by 0.1 N NaOH and the liberated p-nitrophenol was measured at 410 nm. Activity was expressed as a percentage of activity level in absence of metal ions.

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>AP-I Metal</th>
<th>AP-I Metal+EDTA</th>
<th>AP-II Metal</th>
<th>AP-II Metal+EDTA</th>
<th>AP-IV Metal</th>
<th>AP-IV Metal+EDTA</th>
<th>AP-V Metal</th>
<th>AP-V Metal+EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>95.0</td>
<td>100.0</td>
<td>95.0</td>
<td>100.0</td>
<td>96.0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>100.0</td>
<td>87.0</td>
<td>112.5</td>
<td>96.9</td>
<td>102.8</td>
<td>105.9</td>
<td>97.2</td>
<td>105.3</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>25.0</td>
<td>60.9</td>
<td>37.5</td>
<td>92.0</td>
<td>31.9</td>
<td>61.8</td>
<td>15.3</td>
<td>62.3</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0</td>
<td>66.3</td>
<td>0</td>
<td>50.0</td>
<td>6.9</td>
<td>47.0</td>
<td>0</td>
<td>16.7</td>
</tr>
<tr>
<td>Mo⁶⁺</td>
<td>7.0</td>
<td>76.1</td>
<td>31.3</td>
<td>53.6</td>
<td>6.9</td>
<td>41.2</td>
<td>4.2</td>
<td>44.5</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>100.0</td>
<td>100.0</td>
<td>93.8</td>
<td>85.7</td>
<td>105.6</td>
<td>105.9</td>
<td>100.0</td>
<td>109.3</td>
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<tr>
<td>Pb²⁺</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>85.7</td>
<td>72.2</td>
<td>102.9</td>
<td>72.2</td>
<td>111.1</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>20.0</td>
<td>89.1</td>
<td>93.8</td>
<td>85.7</td>
<td>18.0</td>
<td>105.9</td>
<td>12.5</td>
<td>92.6</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>27.0</td>
<td>93.5</td>
<td>25.0</td>
<td>75.0</td>
<td>55.6</td>
<td>108.8</td>
<td>66.7</td>
<td>96.3</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>100.0</td>
<td>65.2</td>
<td>18.7</td>
<td>68.8</td>
<td>129.5</td>
<td>97.0</td>
<td>108.7</td>
<td>88.9</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>100.0</td>
<td>93.5</td>
<td>128.1</td>
<td>100.0</td>
<td>111.1</td>
<td>105.9</td>
<td>100.0</td>
<td>103.7</td>
</tr>
</tbody>
</table>

Table II
Relative rates of Hydrolysis in presence of metal ions
Table 12

Relative rate of Hydrolysis in the presence of inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>AP-I</th>
<th>AP-II</th>
<th>AP-IV</th>
<th>AP-V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alloxan</td>
<td>1 mM</td>
<td>100.0</td>
<td>95.5</td>
<td>79.6</td>
<td>87.5</td>
</tr>
<tr>
<td>NaF</td>
<td>50 mM</td>
<td>22.9</td>
<td>25.0</td>
<td>13.6</td>
<td>10.3</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>50 mM</td>
<td>27.1</td>
<td>25.0</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>Molybdate</td>
<td>1 mM</td>
<td>25.7</td>
<td>23.9</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td>NEM</td>
<td>1 mM</td>
<td>84.3</td>
<td>86.4</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>PCMB</td>
<td>1 mM</td>
<td>54.3</td>
<td>45.7</td>
<td>4.6</td>
<td>5.9</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

The enzymes were incubated with the inhibitors at a desirable concentration for 20 min at 30°C in 0.1 M sodium acetate buffer (pH 5.0). The reaction was started by adding substrate at a final concentration 0.1 mM and incubated for 60 min at 50°C. The reaction was stopped by 0.1 N NaOH and p-nitrophenol liberated was measured at 410 mp. Activity was expressed as a percentage of activity level in absence of inhibitors.
Table 13
Relative rate of hydrolysis in the presence of detergents

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percent</th>
<th>Relative rate of hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AP-I</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.05</td>
<td>117.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>125.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>127.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>123.4</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.1</td>
<td>9.57</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The enzymes were incubated with the substrate at the final concentration of 0.1 mM for 60 min at 50°C in 0.1 M acetate buffer, pH 5.0 after preincubation with detergent for 20 min. The reaction was stopped by adding 0.1 N NaOH and measured the liberated p-nitrophenol at 410 nm. Activity was expressed as a percentage of activity level in absence of detergents.
**Fig. 31**: Determination of optimum time of acid phosphatases
The enzymes were incubated with substrate at a final concentration of 0.1 mM in 0.1 M acetate buffer (pH 5.0) at 37°C for 120 min. The enzyme activity was measured at 410 nm by stopping the reaction with 0.1 N NaOH at each indicated time point.

**Fig. 32**: Determination optimal pH of acid phosphatases
The enzymes were incubated with substrate at a final concentration of 0.1 mM in different pH buffer for 60 min at 50°C. The reaction was stopped by 0.1 N NaOH and the liberated p-nitrophenol was measured at 410 nm.

- •, AP-I; 0 — 0, AP-II; ▲—▲, AP-IV; △—△, AP-V.

**Fig. 33**: Determination of optimum temperature of acid phosphatases
The enzymes were incubated with substrate with a final concentration of 0.1 mM in 0.1 M acetate buffer (pH 5.0) for 60 min at different temperatures. The reaction was stopped by 0.1 N NaOH and the liberated p-nitrophenol was measured at 410 nm.

- •, AP-I; ▲ — ▲, AP-IV; △ — △, AP-V.
AT pH 5.0

Time of Preincubation at 65°C (Minutes)
Percent Activity

100  75  50  25
FIG. 34

AT pH 7.0

Time of Preincubation at 60 °C (minutes)

AT pH 6.0

Percent Activity

Time of Preincubation at 60 °C (minutes)
Figs. 34 
& 35

**Thermal inactivation of acid phosphatases**: Each of the partially purified enzyme proteins were diluted 5 times in 0.1 M acetate buffer (pH 5.0 and pH 7.0) containing 1 mg/ml (final concentration) of BSA. Preincubation was carried out at the indicated temperature for different time periods. The preincubated enzyme protein was taken out at the interval of 10, 20, 30, 45 and 60 min and kept in ice-cold condition. The residual enzyme activity was then determined by incubating with substrate at a final concentration of 0.1 mM in 0.1 M acetate buffer (pH 5.0) for 60 min at 50°C. The reaction was stopped by 0.1 N NaOH and the free p-nitrophenol was measured at 410 nm. Initial activity corresponds to 100 per cent. • — , AP-I ; ○ — ○, AP-II; ▲—▲, AP-IV; △—△, AP-V.
DISCUSSION

Cotyledons from 48-h germinating seeds of V. sinensis contain at least five forms of acid phosphatases, four of which were separated and partially purified by ion-exchange column chromatography and gel filtration.

These phosphatases do, however, have distinctive kinetic and physical properties. These results show that all the phosphatases can function normally at pH 5.0 up to 120 min. AP-I, AP-IV and AP-V have the optimum pH 5.0 while AP-II has the optimum pH 5.5. These values are comparable with the pH optima of total acid phosphatase activity from various plant sources. Optimum activities for plant acid phosphatases have been reported to occur at pH 4.0 in oat seeds (191), between pH 4.7 and 5.0 in wheat roots (192), between pH 5.0 and 6.0 in pea seeds (193) and between 5.2 and 6.0 in rice plants (194). However, the optimum activity of the total acid phosphatase is affected by such factors as the isozymic heterogeneity by the tissues or extracts. Verjee isolated three isozymes of acid phosphatase from wheat germ, whose pH optima ranged from 4.0 and 5.5 on p-nitrophenyl phosphate (195). All the phosphatases show high optimum temperature near about 60°C. These phosphatases differ with respect to their substrate specificity. AP-IV and AP-V are more active than AP-I and AP-II with PEP, ADP and ATP as substrates. AP-I and AP-II show their higher activities on pyrophosphate than that of AP-IV and AP-V. Similar type of higher activity on pyrophosphate was found in acid phosphatase I of Aspergillus saitoi (196). AP-IV and AP-V hydrolyse
2,3-DPG to an appreciable extent, but AP-I and AP-II are completely inactive to this substrate. AP-II hydrolyses α-PGA to a greater extent than that of other phosphatases. So four phosphatases have shown their low activities toward sugar phosphate and high toward nucleotide phosphate. Similar type of substrate specificity was found in the acid phosphatase from the wheat roots (192). The facts indicate that AP-IV and AP-V have the highest activity toward ADP and ATP and these are rather specific for these compounds as suggested by the fact that they may play a role in the energy metabolism of the germinating seeds of *V. sinensis*. Similarly high activity of AP-I on pyrophosphate also suggest that it may play an important role in the metabolism of pyrophosphate.

Ca²⁺ and Mg²⁺ have no effect on the activities of all these phosphatases. Fe³⁺, Hg²⁺, Mo⁶⁺ and Zn²⁺ which are known to inhibit several vegetable phosphatases (13, 193, 197) were found to inhibit the four acid phosphatases of *V. sinensis*, the extent of inhibition being greater with Hg²⁺. Co²⁺ has a strong inhibitory effect on AP-II and slight stimulatory effect on AP-IV. It has no effect on AP-I and AP-V. Similar effect of Co²⁺ on acid phosphatase was found in *Xenopus laevis* Tadpole tails (197) and in wheat roots (192). Mn²⁺ has some stimulatory effect on AP-II and AP-IV. AP-II differs from other three phosphatases with respect to its insensitivity toward Cu²⁺ which was previously reported to be an inhibitor of Tadpole tail acid phosphatase (197). Sodium fluoride, molybdate and orthophosphate, the well known inhibitors of acid phosphatase (196, 198-199), inhibit the activities of all these phosphatases but AP-IV and AP-V are affected
to greater extent. Alloxan, EDTA and NEH have no effect on any of these phosphatase activities. PCMB, which was earlier reported to be an inhibitor of acid phosphatase (198, 200), inhibit the activities of the four phosphatases, but the inhibitory effect is greater in the case of AP-IV and AP-V. These phosphatases are resistant to EDTA and sensitive to PCMB indicating that it is not the metal ion but the cysteine or other sulphydryl group that may participate in the activities of these acid phosphatases.

By the addition of nonionic detergent such as SDS at a concentration of 0.05%, the enzyme activities were inhibited completely. However, in the presence of a nonionic detergent viz. Triton X-100 (0.1%), the activities of AP-I, AP-II and AP-IV increase by 25%, 42% and 12% respectively with no increase in AP-V activity. Since the detergent can disrupt lipoprotein membrane, it is possible that it may release the enzyme from the small membrane fragments.

At pH 5.0 and 7.0 there is no remarkable alternation of AP-II activity at 60°C though there is a striking difference in the denaturation behaviour of AP-I in these conditions. When exposed to 60°C at pH 5.0, the activity of AP-I decreases steadily whereas at pH 7.0, the activity falls suddenly only after 10 min preincubation at 60°C and then remains constant. In contrast, AP-IV activity shows a distinct behaviour at pH 5.0 and pH 7.0 at the same temperature. The activity remains constant at pH 7.0 up to 45 min preincubation, but at pH 5.0, there is a sharp decline in the activity measured at the same temperature. AP-V
loses its activity sharply at pH 7.0 but at pH 5.0 the activity falls gradually.

At still higher temperature viz., at 65°C the activity of AP-I falls rapidly after 10 min preincubation at pH 5.0, then remains constant. Though the activities of AP-II, AP-IV and AP-V follow the same pattern but the activities fall steadily as the time period of preincubation increases. Similar type of thermal inactivation of acid phosphatase from cultured tobacco cells was found by Ueki et al. (201). High thermostability of acid phosphatase from A. saitoi was reported by Naoi et al. (197).
CHAPTER II

SECTION C

COMPARISON OF PHOSPHATASE AND PYROPHOSPHATASE ACTIVITIES

PRESENT IN THE PURIFIED ENZYME FRACTION AP-I
INTRODUCTION

During germination of seeds, different enzymes viz. phosphatase, pyrophosphatase, phytase, etc. which are involved in phosphate metabolism have been found to be markedly changed (119, 193, 201). The possible identity of the phosphomonoesterase and pyrophosphatase in higher plant was suggested by some workers (52, 53), whereas the independent nature of these enzymes in yeast and bacteria has been claimed by others (54, 55). It was found in the previous section that the different isozyme of acid phosphatase has some distinctive properties e.g. substrate specificity, optimum pH, optimum temperature, heat denaturation, metal ions requirement, etc. AP-I enzyme fraction in contrast to other enzyme fractions acts on pyrophosphate as efficiently as on p-nitrophenyl phosphate. Hence it is worth to investigate whether these two activities (acid phosphatase and pyrophosphatase) in AP-I fraction are associated with single protein or different proteins.

On the other hand, there are several reports that a single protein may have two different enzymatic activities e.g. DNase and RNase (202), phosphatase and ATPase (203), alkaline phosphatase and alkaline pyrophosphatase (204, 205), acid phosphatase and acid pyrophosphatase (206), phosphatase and mutase (207). The different identity of lysosomal acid phosphatase and acid pyrophosphatase have also been reported by Brightwell (208). The present section is mainly devoted to the comparative studies on phosphatase and pyrophosphatase activities present in AP-I fraction.
Purchase and collection of all the materials required in the experiments were mentioned in the Sections A and B of Chapter II.

METHODS

Enzyme source used in the experiment was the purified enzyme fraction AP-I. Acid pyrophosphatase activity was assayed by incubating the enzyme with neutral pyrophosphate at pH 5.0 (acetate buffer) in a total volume of 1 ml. Final concentration of pyrophosphate and acetate were 0.1 mM and 10 mM respectively. After incubation at 50°C for 60 min sample was deproteinized by 10% TCA and the liberated orthophosphate was determined according to the method as described by Lowry and Lopez (113). Usually the amount of protein present in the enzyme preparation used was so small that no separation of protein by precipitation and centrifugation was necessary as it did not interfere in the subsequent phosphate determination.

Acid phosphatase activity was assayed by incubating the enzyme sample with p-nitrophenyl phosphate at pH 5.0 for 60 min at 50°C in a total volume of 1 ml. Final concentration of substrate and acetate were 0.1 mM and 10 mM respectively. Reaction was stopped by 0.1 N NaOH for p-nitrophenol and by 10% TCA for phosphate determination.
Gel filtration through Sephadex G-150:

The partially purified enzyme fraction (AP-1) was concentrated by dialysis against PEG and then against 10 mM Tris-HCl buffer (pH 7.4) to remove PEG. 2.0 ml of this concentrated sample was applied onto a Sephadex G-150 gel (85 x 1 cm) which was equilibrated previously with 10 mM Tris-HCl buffer (pH 7.4). Elution was performed with the same buffer at a flow rate of 6.0 ml/h and fractions of 2.0 ml were collected. Fig. 36 shows the elution profile of the enzyme protein through Sephadex G-150.

Heat Inactivation:

AP-1 was diluted five times in 0.1 M acetate buffer (pH 5.0). BSA was added to the enzyme solution at a final concentration of 1 mg/ml. Preincubation was carried out at the indicated temperature given in the legend of Fig. 38. At different time interval aliquots were removed and assayed for acid phosphatase and acid pyrophosphatase activities at 50°C.

Energy of Activation:

At pH 5.0 (0.1 M acetate buffer), the reaction velocity was determined as the function of temperature over the range 30°C to 50°C for phosphatase and pyrophosphatase activities using p-nitrophenyl phosphate and pyrophosphate as substrates. After 30 min incubation the reaction was stopped by 10% TCA and the free phosphate was estimated according to the method as described by Lowry and Lopez (113).
Elution of the enzyme protein (AP-1 fraction) having phosphatase and pyrophosphatase activities through Sephadex G-150 gel started from fraction number 17 and reached a peak at fraction 22 followed by a sharp fall. The ratio of the two activities throughout the peak fractions obtained from Sephadex G-150 gel filtration was essentially constant, varying only from 1.0 to 2.0 (Fig. 36).

Optimum pH:

Both the activities of phosphatase and pyrophosphatase present in AP-I fraction were studied at different pH (3.0 to 9.0) using a variety of buffers. Acid phosphatase and pyrophosphatase activities at the different pH values are shown in Fig. 37A. Acid phosphatase was high between 4.0 to 5.5 with the maximum activity between pH 5.0 to 5.5. No phosphatase activity was observed in neutral to alkaline pH. However, pyrophosphatase activity was high over a broad pH range viz., pH 4.0 to 7.0, showing a peak at pH 5.0 while in the alkaline range the activity was quite low.

Optimum temperature:

Acid phosphatase activity in AP-I fraction had an optimum temperature which was about 60°C. The temperature optima of pyrophosphatase activity was also 60°C (Fig. 37B).
Heat Denaturation:

Fig. 38 shows the thermal inactivation profiles of acid phosphatase activity and pyrophosphatase activity in AP-I fraction. The enzyme protein was heated at a fixed temperature for different periods of time. At 60°C both the activities were found to be decreased with the increase in time of incubation. Of the initial enzymatic activity, 50% was retained in case of acid phosphatase and 70% in case of pyrophosphatase after 45 min of incubation at 60°C in acetate buffer (pH 5.0). Upon incubation at 65°C for 45 min in the same buffer, acid phosphatase activity retained 67% of its initial activity and pyrophosphatase activity retained 68% of its initial activity.

Effects of metal ions:

The effects of various metal ions, known to inhibit the several pyrophosphatase and acid phosphatase activities were studied to find the differences in the behaviour of the two enzyme activities present in AP-I fraction. The metal ions were added to the buffer-enzyme mixture at a final concentration of 1 mM before adding the substrate. The results are given in Table 1. Mg²⁺ had inhibitory effect on acid pyrophosphatase activity up to 20% while it had no any effect on acid phosphatase activity. Both the activities of acid phosphatase and pyrophosphatase were inhibited by Mo⁶⁺. Pyrophosphatase activity was greatly inhibited by Hg²⁺, Pb²⁺, Co²⁺, Mn²⁺ and Zn²⁺. There were some stimulatory effects of EDTA on pyrophosphatase activity present in the system containing Mn²⁺ and Zn²⁺. Though acid phosphatase activity was
unaffected by Mn\textsuperscript{2+}, Pb\textsuperscript{2+} and Co\textsuperscript{2+}, its activity was affected by Hg\textsuperscript{2+} and Zn\textsuperscript{2+}. Both the activities were found to be inhibited by Fe\textsuperscript{3+}. But the inhibitory effect of Fe\textsuperscript{3+} on the acid phosphatase activity could be nullified partially by EDTA whereas no such reversal was observed in the case of pyrophosphatase activity. Cu\textsuperscript{2+} was found to have an inhibitory effect on acid phosphatase activity whereas Ca\textsuperscript{2+} had no effect on any of the enzymatic activities.

Effect of inhibitors:

Table 15 shows the effects of some well-known inhibitors of enzyme protein. Sodium fluoride, sodium molybdate and ascorbic acid inhibited both the activities of acid phosphatase and acid pyrophosphatase but acid phosphatase activity was more affected by these inhibitors. PCMB inhibited both the activities whereas NEM had no inhibitory effect. Tartrate had little inhibitory effect on acid phosphatase activity and acid pyrophosphatase activity.

Inhibition of acid phosphatase activity present in AP-I fraction by Pyrophosphate:

It was found in the previous section of this chapter that pyrophosphate was a potent inhibitor of acid phosphatase activity. The type of inhibition by pyrophosphate and the inhibitor constant were determined from the initial velocity measurement at various substrate concentrations (0.05 mM, 0.1 mM, 0.2 mM and 0.3 mM) in the presence of varying inhibitor concentrations (0.5 mM, 1.0 mM...
and 2.0 mM). The results have been presented according to Lineweaver-Burk plot. The common intercept on the ordinate (1/v axis) indicated that the inhibition of acid phosphatase activity by pyrophosphate was competitive in nature. The $K_i$ values obtained from secondary plot (given in inset) was about $8 \times 10^{-4}$M. The result was presented in Fig. 39.

Energy of activation:

At optimum pH (5.0) the acid phosphatase and acid pyrophosphatase activities were determined at different temperatures (30°C, 40°C and 50°C). The incubation period was 30 min for all the cases. The logarithms of velocities of hydrolysis were plotted against the reciprocals of the absolute temperature (1/T) as shown in Fig. 40. From the slopes of the lines the energy of activation were calculated. The values obtained for acid phosphatase and acid pyrophosphatase activities were found to be 10.3 and 10.9 Kcal/mole respectively.
Table 14

Effects of metal ions and EDTA on acid phosphatase and acid pyrophosphatase activities present in the AP-I fraction

<table>
<thead>
<tr>
<th>Metal given</th>
<th>Acid phosphatase</th>
<th>Acid pyrophosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent of activity retained</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metal</td>
<td>Metal + EDTA</td>
</tr>
<tr>
<td>-</td>
<td>100.0</td>
<td>98.0</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>100.0</td>
<td>93.0</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>100.0</td>
<td>87.0</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>25.0</td>
<td>60.9</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>0</td>
<td>66.3</td>
</tr>
<tr>
<td>Mo$^{6+}$</td>
<td>7.0</td>
<td>76.1</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Pb$^{2+}$</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>20.0</td>
<td>89.1</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>27.0</td>
<td>93.2</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>*100.0</td>
<td>*65.2</td>
</tr>
</tbody>
</table>

* Results obtained from repeated experiments.

The enzyme was incubated with substrate and metal ions at the final concentrations of 0.1 mM and 1.0 mM respectively for 60 min at 50°C in 0.1 mM sodium acetate buffer (pH 5.0). The metal ions were added to the buffer-enzyme mixture 20 min before adding the substrate. In case of metal-EDTA systems enzyme proteins were preincubated with metal ions for 20 min followed by another 10 min preincubation with EDTA. The reaction was terminated by the addition of TCA to a final concentration of 5%. The amount of liberated phosphate was measured by the method described by Lowry and Lopez (113). Activity was expressed as a percentage of activity level in absence of metal ions.
Effects of different inhibitors on acid phosphatase and acid pyrophosphatase activities present in AP-I fraction

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration (mM)</th>
<th>Percent of activity retained</th>
<th>Acid phosphatase</th>
<th>Acid pyrophosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>NaF</td>
<td>50</td>
<td>20.0</td>
<td>45.0</td>
<td></td>
</tr>
<tr>
<td>Molybdate</td>
<td>1</td>
<td>20.0</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>NEM</td>
<td>1</td>
<td>100.0</td>
<td>90.0</td>
<td></td>
</tr>
<tr>
<td>PCMB</td>
<td>1</td>
<td>60.0</td>
<td>70.0</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>5</td>
<td>40.0</td>
<td>70.0</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>5</td>
<td>100.0</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>Tartrate</td>
<td>5</td>
<td>80.0</td>
<td>80.0</td>
<td></td>
</tr>
</tbody>
</table>

The enzyme was incubated with the inhibitors at the specified concentrations for 20 min at 30°C in 0.1 M sodium acetate buffer (pH 5.0). The reaction was started by the addition of respective substrates at a final concentration of 0.1 mM and incubated at 50°C for 60 min. The reaction was stopped by the addition of TCA to a final concentration of 5%. The amount of released phosphate was measured by the method of Lowry and Lopez (113). All values were expressed as the per cent of amount of phosphate released in the absence of inhibitors.
FIG. 38

Percentage of activity remained

Time of Preincubation at pH 5.0

at 60°C

Acid pyrophosphatase

Acid phosphatase

at 65°C

Acid pyrophosphatase

Acid phosphatase
FIGURE LEGENDS

Fig. 36: Sephadex G-150 gel filtration of acid phosphatase and pyrophosphatase activities present in AP-I fraction.
This enzyme fraction (AP-I) was dialysed against PEG to concentrate the protein and against 10 mM Tris-HCl buffer (pH 7.4) to remove the PEG. This concentrate fraction (2.0 ml) containing 0.05 mg of protein was loaded onto a Sephadex gel (85 x 1 cm) which was equilibrated previously with 10 mM Tris-HCl buffer (pH 7.5). Elution was performed with the same buffer at a flow rate of 6 ml/h and fractions of 2.0 ml were collected. The acid phosphatase activity and acid pyrophosphatase activity in each fraction was assayed by measuring the liberated orthophosphate and plotted against the fraction number.

Fig. 37A: Determination of optimum pH of acid phosphatase and acid pyrophosphatase activities present in AP-I fraction.
The acid phosphatase and pyrophosphatase activities were assayed by incubating the enzyme fraction (AP-I) with respective substrates at a final concentration of 0.1 mM at different pH for 60 min at 30°C. The reaction was stopped by 10% cold TCA and the liberated orthophosphate was measured according to the method described by Lowry and Lopez (113).
Fig. 37B: Determination of optimum pH of acid phosphatase and acid pyrophosphatase activities present in AP-I fraction:
The acid phosphatase and acid pyrophosphatase activities were assayed by incubating the enzyme fraction with respective substrate at a final concentration of 0.1 mM in 0.1 M acetate buffer (pH 5.0) for 60 min at different temperature, the reaction was stopped by 10% cold TCA and the liberated orthophosphate was measured according to the method as described by Lowry and Lopez (113).

Fig. 38: Thermal inactivation of acid phosphatase and pyrophosphatase activities present in AP-I fraction:
The enzyme protein (AP-I) was diluted 5 times in 0.1 M acetate buffer (pH 5.0) containing BSA (1 mg/ml, final concentration). Preincubation was carried out at the indicated temperature for different time periods. The preincubated enzyme protein was taken out at the interval of 10, 20, 30 and 45 min and kept in ice-cold condition. The residual enzyme activities were determined after 60 min incubation with respective substrate at a final concentration of 0.1 mM in 0.1 M acetate buffer (pH 5.0) at 50°C. The reaction was stopped by 10% cold TCA and the liberated orthophosphate was measured according to the method as described by Lowry and Lopez (113). Initial activity was taken as 100 per cent.
Fig. 39: Inhibition of acid phosphatase activity by pyrophosphate.

The reaction was carried out in acetate buffer (pH 5.0) by incubating the reaction mixture at 50°C for 60 min and the liberated p-nitrophenol was measured at 410 nm.

- o—o, No inhibitor; 0.05 mM pyrophosphate;
- △—△, 1.0 mM pyrophosphate;
- x—x, 2.0 mM pyrophosphate.

Fig. 40: Effect of temperature on the rate of hydrolysis of p-nitrophenyl phosphate and pyrophosphate by acid phosphatase and pyrophosphatase which were associated with AP-I fraction. Experiments were done at 30°, 40° and 50°C by incubating the enzyme protein with respective substrates for 30 min in acetate buffer.

Curve A, hydrolysis of p-nitrophenyl phosphate;
Curve B, hydrolysis of pyrophosphate.
DISCUSSION

The AP-I fraction hydrolyses pyrophosphate and p-nitrophenyl phosphate at nearly the same rate. From the gel filtration profile of the enzyme protein it is apparent that the two activities are eluted in the same way. The ratio of the two activities throughout the peak is near about same. Both the activities exhibit optimum pH at 5.0. However, the acid phosphatase activity is lost at pH 7.0 onward but low pyrophosphatase activity is found upto pH 9.0. The optimum temperature for both the activities is 60°C. In between 37°C to 60°C, the reaction exhibits a Q10 ranging from 0.8 to 1.0. At 60°C the rate is approximately 3-4 times than that found at 37°C, but above 60-65°C the rate quickly falls off.

The residual activity observed above 65°C may have reflected limited enzymatic activity before the reaction mixture achieved thermal equilibrium. Acid phosphatase loses 50% of its activity at 60°C for 10 min incubation whereas acid pyrophosphatase retains 70% of its activity. From this result it may be suggested that the enzyme fraction AP-I contains some heat stable component which has the greater pyrophosphatase activity. This suggestion is further corroborated with another result of heat stability at 65°C. At 65°C the pyrophosphatase activity in AP-I fraction loses 30% of its activity upon 10 min incubation. But it does not lose its activity on further incubation. Similar result is also obtained in case of acid phosphatase with the loss of 55% of its activity.

There are distinct similarity and dissimilarity between
the acid phosphatase and pyrophosphatase activities in their behaviour toward divalent and heavy metal ions. Mg$^{2+}$, a well known activator of alkaline phosphatase (209,210) and alkaline pyrophosphatase (211) has inhibitory effect on the acid pyrophosphatase activity but has no effect on the acid phosphatase activity. The inhibitory effect of Mg$^{2+}$ on acid pyrophosphatase was also reported by Naganna et al. (50). Mo$^{6+}$, which is known to inhibit several vegetable and animal tissue phosphatases (55) is found to inhibit both the activities. Ca$^{2+}$, Hg$^{2+}$ and Zn$^{2+}$ have some inhibitory effects on the acid phosphatase and pyrophosphatase activities. But these two activities differ distinctly with respect to their sensitivity toward Mn$^{2+}$, Pb$^{2+}$, Co$^{2+}$ and Cu$^{2+}$. Acid phosphatase activity is unaffected by Mn$^{2+}$, Pb$^{2+}$ and Co$^{2+}$, while pyrophosphatase activity is inhibited by the three metal ions, the extent of inhibition being greater with Mn$^{2+}$. The inhibitory effect of Mn$^{2+}$ and Co$^{2+}$ on pyrophosphatase activity but not on phosphatase activity was also reported by Naganna et al. (50). In contrast, Cu$^{2+}$ shows two opposite effect on the two enzyme activities. Acid phosphatase activity is inhibited by Cu$^{2+}$ whereas pyrophosphatase activity is slightly higher in the presence of Cu$^{2+}$. Fe$^{3+}$ inhibits both the activities, but it is found from the result that Fe$^{3+}$ is more potent inhibitor of pyrophosphatase than that of phosphatase. The two activities in presence of metal ions also differ markedly with respect to the chelating effect of ethylene diamine tetra-acetic acid. In presence of EDTA and Mn$^{2+}$ there is some stimulation (23%) of acid pyrophosphatase activity. Similarly Zn$^{2+}$ also stimulates the pyrophosphatase activity. But in case of Co$^{2+}$ and EDTA, acid phosphatase activity is inhibited.
These paradoxical results may be due to the fact that minute amount of metal ions is essential for the enzyme which is attained in the assay mixture after the metal-EDTA interaction. Attempts to find any stimulation of the enzyme activity with minute amount of these metal ions as was obtained with yeast pyrophosphatase by Kunitz (55) have failed. However, Naganna (50) also tried to find out any effect of minute amount of metal ions on potato pyrophosphatase activity but no activation was observed by Co²⁺, Zn²⁺ and Mn²⁺. Due to various side effects of metal ions (substrate precipitation, complex formation, pH change, etc.), it is very difficult to compare the phosphatase and pyrophosphatase activities to their behaviour toward these metal ions.

Sodium fluoride and molybdate, the well known inhibitors of phosphatase (196, 198) inhibit both the activities. Acid phosphatase is more affected. Ascorbic acid also inhibits the phosphatase and pyrophosphatase activities. p-Chloromercuric benzoate which is an inhibitor of many enzymes (200) inhibits both the activities. Both the activities show almost equal sensitivity towards the inhibitors.

Addition of pyrophosphate to the reaction mixture containing p-nitrophenyl phosphate as a substrate resulted in the inhibition of hydrolysis of p-nitrophenyl phosphate, the extent of inhibition depending upon the concentration of enzyme, p-nitrophenyl phosphate and pyrophosphate. The Lineweaver-Burk plot shows that pyrophosphate is a competitive inhibitor of p-nitrophenyl phosphate. The competitive type of inhibition of acid phosphatase activity from Havrea brasiliensis Latex by pyrophosphatase was
The mechanism of inhibition would be complex since both p-nitrophenyl phosphate and pyrophosphate are the good substrates of acid phosphatase. By the degradation of the inhibitor (pyrophosphate), particularly at low p-nitrophenyl phosphate concentration, an appreciable amount of phosphate produced which might also be an inhibitor.

The activation energy for the hydrolysis of p-nitrophenyl phosphate and pyrophosphate by AP-I enzyme fraction are 10.3 Kcal/mole and 10.94 Kcal/mole respectively. For glycerophosphate hydrolysis by bone phosphatase, a value of 9.34 Kcal/mole was reported by Bodansky (212), 9.8 Kcal/mole for the hydrolysis of pyrophosphate was found by Naganna (50) in potato enzyme respectively. From these results it is evident that the values reported here are in very close agreement with the values reported by Bodansky and Naganna. It is also of interest that there is a close resemblance between the values of activation energy for the hydrolysis of pyrophosphate and p-nitrophenyl phosphate.

It has been pointed out earlier that there are many enzyme systems which have different catalytic activities within a same enzyme population. AP-I fraction has high phosphatase and pyrophosphatase activities. One may conclude that the pyrophosphatase activity in AP-I fraction might be due to some impurity, but the evidence here favours the common identity of phosphatase and pyrophosphatase activities. The data from the comparison of phosphatase and pyrophosphatase activities on the whole support the idea that these two activities are associated with the same enzyme. The pH optimum, temperature optimum and metal ions effects
also suggest the same notion. The results of heat inactivation, effects of some inhibitors and energy of activation are also consistent. Pyrophosphate inhibits the acid phosphatase activity competitively. The simplest satisfactory explanation of this observation is that the two compounds are substrates for the same enzyme and compete for the same active site. The column chromatography techniques failed to achieve any separation of the two activities and there is good colinearity in the elution behaviour of these two activities.

The microheterogeneity in the properties of the two activities may reflect their differences in the nonfunctional part of the enzyme protein present in the population of a single enzyme system.

All these observations support the idea that phosphatase and pyrophosphatase activities are probably associated with the same enzyme protein. This enzyme is nonspecific acid phosphatase and is responsible for the hydrolysis of pyrophosphate. Other phosphomonoesterases have been described as possible pyrophosphatases. For instance, Moss et al. (213) reported that alkaline phosphatases from a variety of mammalian tissues are all active pyrophosphatases, and Nordlie and Arion (214) consider that a liver microsomal glucose-6-phosphatase is also a pyrophosphatase. Mayer et al. (53) found that a soybean acid phosphatase is probably a pyrophosphatase. Purified potato acid phosphatase has considerable pyrophosphatase activity (65). Other plant phosphatases with claims to pyrophosphatase activity include phosphatase from Pisum sativum seedlings (215), from whitelupin...
seedlings (52) and from rice ears and apricot kernels (58).

The biological significance of the single enzyme protein which serves the multiple role in the biological system offers some economic importance and vital functions in the living systems.