Partial purification and some biochemical properties of non-specific alkaline phosphatase in germinating chick-pea (Cicer arietinum) seeds

Trinidad Angosto and Angel Matilla


A procedure for the partial purification of a non-specific alkaline phosphatase (EC 3.1.3.1) from the embryonic axes of chick-pea seeds is described. Ammonium sulphate precipitation, DEAE-cellulose chromatography, Sephacryl S-200 chromatography and polyacrylamide gel electrophoresis are the most important steps. The molecular weight of this non-specific enzyme, as determined by Sephacryl S-200 gel filtration and SDS-polyacrylamide gel electrophoresis, was estimated as being 68 and 78 kDa respectively; the optimum pH for p-nitrophenylphosphate hydrolysis was 7.5, and the $K_m$ for this artificial substrate was 0.5 mM. The enzyme catalyzes the hydrolysis of a variety of organic phosphate esters. The best substrates are: phosphoenolpyruvate ($K_m=2.4$ mM), NADP$^+$ ($K_m=4.0$ mM), 5'-AMP ($K_m=4.5$ mM), 5'-ADP ($K_m=6.1$ mM) and ribose-5P ($K_m=5.8$ mM); but it is unable to hydrolyze 5'-ATP, phosphocreatine and triphosphophate. Phosphate was a competitive inhibitor. Zn$^{2+}$, K$^+$, Hg$^{2+}$ and Mo$^{6+}$ were strong inhibitors, whereas F$^-$ and Ca$^{2+}$ inhibited weakly; Co$^{2+}$ and Ni$^{2+}$ were activators.

Key words – Alkaline phosphatase, Cicer arietinum L. cv. Castellana, embryonic axes, germination, seed, seedling growth.

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Introduction

Phosphorus is essential for the growth and development of organisms and a lack of this element is one of the factors that limit the growth of plants and in particular the development of its organs. Plants obtain phosphorus from the soil (Mengel and Kirkby 1987), from phytine (Bartnik and Szafrańska 1987) or from other phosphorylated molecules and store it in their cell vacuoles (Bielecki and Ferguson 1983). Non-specific phosphatases are involved in the cell metabolism of phosphorus. These enzymes act on a great variety of phosphorylated substrates at different rates, which characterizes them as non-specific (Hollander 1971).

The alkaline phosphatases (AlPases; EC 3.1.3.1) are a group of phosphomonoesterases which catalyze the hydrolysis of phosphate esters, releasing inorganic phosphorus. They are widely distributed along the evolutionary scale, and although their function has been studied in microorganisms and eukaryotic animals (Stadtman 1961, Reid and Wilson 1971) little data exist about their role in higher plants. In some cases it has not been possible to quantify or even detect them in plants (Hota and Israelstam 1978).

In this paper we describe the presence of an AlPase in Cicer arietinum seeds and discuss some of its biochemical properties.

Abbreviations – AlPase, alkaline phosphatase; $K_m$, Michaelis constant; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; $p$-NPP, $p$-nitrophenylphosphate; SDS, sodium dodecyl sulphate.

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Materials and methods

Plant material

The experiments were conducted with seeds of *Cicer arietinum* L. cv. Castellana, harvested in 1985. They were germinated for 24 h in darkness at 25°C on moist filter paper in Petri dishes at a relative humidity of 70%. The testa was then removed and the embryonic axes separated from the cotyledons.

Preparation of enzyme extract

The cotyledons and embryonic axes (10 g) were homogenized separately in a mortar for 10 min at 0-4°C in 20 ml 25 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂. The homogenates were centrifuged at 30000 g (4°C) for 30 min in a refrigerated Sorvall RC-5 centrifuge. The supernatant was used as crude extract for the determination and partial purification of AlPase.

Assay of AlPase activity

AlPase activity was measured spectrophotometrically determining the increase in absorbance at 410 nm due to the release of p-nitrophenol from p-NPP. The assay mixture (0.5 ml) contained 20 mM Tris-HCl (pH 7.5), 30 mM p-NPP and enzyme extract and was incubated for 5 min at 28°C. The reaction was started by the addition of substrate and stopped with 500 mM NaOH (0.5 ml). One unit of AlPase activity was defined as the amount of enzyme catalyzing the release of one μmol of p-NPP per min compared to a control without substrate.

When natural substrates were used the rate of hydrolysis was calculated from the quantity of mineral phosphate formed during the experiment. Phosphorus was determined spectrophotometrically at 820 nm, using molybdenum blue obtained from the phosphomolybdate complex and with ascorbic acid as reducer, according to the method of Ames (1966).

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

**Fig. 1.** Variation of AlPase activity (×-×) and embryonic axes (○-○) during germination of chick-pea seeds. Vertical bars are SE of 3-4 series.

Purification of AlPase

AlPase was purified from the crude extract obtained from the embryonic axes of chick-pea seeds after 24 h germinating in distilled water. Ammonium sulfate was added slowly to the crude extract to 40% saturation at 4°C, stirred gently for 30 min and centrifuged at 6000 g for 30 min. The precipitate was discarded and the resulting supernatant was brought to 70% saturation by the addition of ammonium sulfate, stirred and centrifuged as before. The sedimented material was collected, suspended in a minimum volume of 20 mM Tris-HCl buffer (pH 7.5), and dialyzed for 12 h against the same solution.

The dialyzed material was subjected to ion-exchange

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (μmol p-NPP mg⁻¹ min⁻¹)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant*</td>
<td>300</td>
<td>8</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>40% (NH₄)₂SO₄ supernatant</td>
<td>196</td>
<td>14</td>
<td>3.3</td>
<td>100</td>
</tr>
<tr>
<td>70% (NH₄)₂SO₄ precipitate</td>
<td>113</td>
<td>19</td>
<td>4.7</td>
<td>91</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>13.1</td>
<td>42</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>1.6</td>
<td>167</td>
<td>41</td>
<td>11</td>
</tr>
</tbody>
</table>

Tab. 1. Summary of the purification procedure of alkaline phosphatase from chick-pea seeds. Results are based on 10 g fresh weight of embryonic axes. Enzyme activity was assayed using p-NPP as the artificial substrate. *Refers to crude extract.
chromatography on a DEAE-cellulose column (2.5 × 25 cm) equilibrated with 20 mM Tris-HCl buffer. After washing with 50 ml of buffer the column was eluted with a 300 ml linear gradient from 0 to 1.0 M NaCl at a constant flow of 30 ml h⁻¹, and fractions of 5 ml were collected. The active fractions were pooled and concentrated against PEG 6000 or lyophilized. The concentrate was chromatographed on a Sephacryl S-200 column (Pharmacia Fine Chemicals) equilibrated with 10 mM Tris-HCl (pH 8.0). Elution with the same buffer was carried out at a constant flow of 43 ml h⁻¹ and fractions of 3 ml were collected. The active fractions were pooled, concentrated as above and stored at −10°C with no loss of activity.

**Protein determination**

Protein was determined according to Bradford (1976), using bovine serum albumin as standard. The relative protein concentrations in the effluents from the columns were determined as the absorbance at 280 nm.

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**Fig. 2.** DEAE-cellulose column chromatography of AlPase. Experimental details are described in Materials and methods. Absorbance at 280 nm of each fraction (●●), specific activity (○○).

**Fig. 3.** Sephacryl S-200 column chromatography of AlPase. Active fractions of DEAE-cellulose chromatography were passed through Sephacryl S-200. Experimental details are described in the text. Absorbance at 280 nm of each fraction (●●), absorbance at 410 nm (○○) following phosphatase assay as in text, specific activity (△△).

**Fig. 4.** PAGE under non-denaturing conditions of the purified AlPase. Electrophoresis was carried out in a 7.5% gel at pH 8.0, and the gel was stained with Coomassie blue R.
Fig. 5. SDS-PAGE and apparent molecular weight determination of AlPase (12.5 µg sample) from \textit{Cicer arietinum}. Molecular weights of the marker proteins were: 1. Trypsin inhibitor (20.1 kDa); 2. lactate dehydrogenase (36.5 kDa); 3. Glutamate dehydrogenase (55.4 kDa); 4. The AlPase from chick-pea seeds (estimated molecular weight 78 kDa); 5. Phosphorylase b (97.4 kDa) and 6. \(\alpha\)-microglobulin (170.0 kDa). \(K_\text{av}\) represents the fractions of the stationary gel volume available for diffusion of a given solute species.

**PAGE and determination of molecular weight**

PAGE was performed under non-denaturing conditions as described by Davis (1964). The molecular weight of native AlPase was determined by gel filtration through a column of Sephacryl S-200 as described above; the protein molecular weight standards used were cytochrome-c (12.4 kDa), carbonic anhydrase (29.0 kDa), acid phosphatase of chick-pea seed (39 kDa), bovine serum albumin (68.0 kDa) and E. coli AlPase (80.0 kDa). PAGE was also performed under denaturing conditions by using SDS (Laemmli 1970) and standard proteins of known molecular weight. Densitometry was performed at 500 nm with a spectrophotometer equipped with a gel scanner. Gels were stained for protein with 0.25% (w/v) Coomassie blue R in 7% (v/v) acetic acid and 40% (v/v) methanol.

**Results and Discussion**

**Variation in the AlPase activity during germination**

The change in activity of the enzyme was investigated during the first 48 h of germination prior to studying its biochemical properties (Fig. 1). This was done in order to determine its involvement in the germination process, since AlPase is an important component in cell metabolism and especially in germination (Guardioli and Sutcliffe 1971). The specific activity in the cotyledon varied.

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**Fig. 6.** Apparent molecular weight of AlPase from \textit{Cicer arietinum} as determined by gel filtration on Sephacryl S-200. Molecular weights of the marker proteins were: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), acid phosphatase of chick-pea seed (39 kDa), and BSA (68 kDa). \(K_\text{av}\) as in Fig. 5.

**Fig. 7.** Effect of pH on the activity of the AlPase. The partially purified enzyme (5 µg) was assayed as described in Materials and methods in 10 mM sodium acetate buffer (××), 10 mM Tris-HCl buffer (○○), and 10 mM glycine-NaOH buffer (●●) using 30 mM p-NPP as substrate. Vertical bars denote ± se of 2–3 series.
Tab. 2. Substrate specificity of chick-pea seed alkaline phosphatase. The concentration of each substrate for determination of $V_{\text{max}}$ (nmol P$_i$ min$^{-1}$) was 3 mM whereas for $K_m$ concentrations from 0.5 mM to 10 mM were used with 5 μg of partially purified protein. Means ± se (n=3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$ (mM)</th>
</tr>
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<tbody>
<tr>
<td>p-NPP</td>
<td>70±7</td>
<td>0.5</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>32±3</td>
<td>2.4</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>19±5</td>
<td>4.5</td>
</tr>
<tr>
<td>5'-ADP</td>
<td>14±3</td>
<td>6.1</td>
</tr>
<tr>
<td>5'-ATP</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>NADP</td>
<td>19±2</td>
<td>4.0</td>
</tr>
<tr>
<td>Ribose-5P</td>
<td>14±2</td>
<td>5.8</td>
</tr>
<tr>
<td>Fructose-1,6dP</td>
<td>10±3</td>
<td>7.6</td>
</tr>
<tr>
<td>Fructose-6P</td>
<td>6±1</td>
<td>12.5</td>
</tr>
<tr>
<td>Glucose-6P</td>
<td>8±1</td>
<td>11.3</td>
</tr>
<tr>
<td>Glucose-1P</td>
<td>3±1</td>
<td>25.0</td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Triphosphatine</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

between 12 and 18 μmol p-NPP min$^{-1}$ (mg fresh weight)$^{-1}$, increasing with the germination time. Activity in the embryonic axes was 2.3 times greater at 48 h than at 6 h (Fig. 1). These findings lead us to conclude that this enzyme activity is related to the germination process. One possible explanation for the larger increase in AlPase activity in the embryonic axis than in the cotyledon may be that the reserve tissue already contains a large concentration of soluble P$_i$ (approximately 100 times as much as the cotyledon; Angosto et al. 1988). During the life cycle of Blastocladia emersoni, AlPase decreased slightly during germination but increased about 4.5-fold during sporulation (Selitrennikoff and Sonneborn 1977).

Fig. 9. Phosphate inhibition of the hydrolysis of the p-NPP by the purified AlPase. The experimental conditions were those of the standard assay (see Materials and methods). The activity without phosphate was as in Tab. 3. Bars are ± se of 2–3 series.

Purification of AlPase

Table 1 shows the yields and specific activities obtained in the purification steps. The AlPase was purified about 41 times from the original crude extract with an overall recovery of 11%.

On DEAE-cellulose chromatography, AlPase was eluted at 0.3 M NaCl as a single and well-defined peak (Fig. 2). Fractions 24–27 were combined; with this step the protein content decreased 8 times and the specific activity increased 2 times; the purification factor was 10 (Tab. 1). The concentrated fractions (24–27) were run on a Sephacryl S-200 column (Fig. 3) and the active fractions collected and concentrated. The final preparation had a specific activity 4 times higher than after the DEAE-cellulose step and the purification factor was 41 (Tab. 1). When the enzyme was analyzed by PAGE a single band of protein was observed in both the native (Fig. 4) and denatured states (Fig. 5).

Tab. 3. Effect of metal ions and some anions on alkaline phosphatase activity. The activity of the purified enzyme (5 μg) was assayed in the presence of various substances at a concentration of 5 mM and expressed as percentage of the control activity (166.7 ± 13.0 μmol p-NPP mg$^{-1}$ min$^{-1}$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>15</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>103</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>77</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>25</td>
</tr>
<tr>
<td>K$^+$</td>
<td>15</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>125</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>105</td>
</tr>
<tr>
<td>Ammonium</td>
<td>70</td>
</tr>
<tr>
<td>Molybdate</td>
<td>26</td>
</tr>
<tr>
<td>Fluoride (1 mM only)</td>
<td>80</td>
</tr>
</tbody>
</table>

Fig. 8. Effect of protein concentration (A) and incubation time (B) on the AlPase activity. Incubation time (A) and protein concentration (B) were 5 min and 5 μg, respectively. Vertical bars denote ± se of 3–4 series.
Molecular weight

The native molecular weight of the AlPase was 68 ± 5 kDa (n=3) as determined by gel filtration on Sephacryl S-200 (Fig. 6). However, when the final preparation was denatured and subjected to SDS-PAGE, a single protein staining band migrating with a molecular mass of about 78 kDa was observed (Fig. 5). There was no sign of the presence of subunits. Values reported for other organisms are: 50 kDa (monomeric) in both *Candida tropicalis* (Bedu et al. 1982) and *Spirodea oligorrhiza* (Bieleski 1974), 60 kDa (monomeric) in *Vibrio cholerae* (Roy et al. 1982), 160 kDa (monomeric) in *Anacystis nidulans* R2 (Block and Grossman 1988), 673 kDa (dimeric) in *Pythococcus blakesleeanus* (Cohen 1985) and 120 kDa (dimeric) in *Penicillium chrysogenenum* (Kliment et al. 1987).

Biochemical properties of the AlPase

The optimum pH was determined at 28°C using buffers from pH 4.0 to 10.0 and p-NPP as the artificial substrate. The buffers used were sodium acetate for pH 5.0 to 6.0, Tris-HCl for pH 6.0 to 8.0 and glycine-NaOH for pH 8.0 to 10.0. Maximum activity was at about pH 7.5 (Fig. 7). Therefore the presence of a light shoulder at pH 6.0 could be indicative of a small acid phosphatase contamination as described by Bieleski (1974). This contamination could explain the residual activity of AlPase on substrates of chick-pea acid phosphatase (Anastasio et al. 1988). The optimum pH for other non-specific alkaline phosphatases are 7.5 for *Spirodea oligorrhiza* (Bieleski 1974), 8.0 for *Saccharomyces* (Onishi et al. 1979), 9.8 for *Saccharomyces bicolour* (Rajasekhar and Sopory 1985), 8.2 for *Vibrio* (Woolkalis and Baumann 1981), and 11.0 for *Bacillus* sp. OK-1 (Nomoto et al. 1988)

The *Kₐ* values for several phosphorylated molecules were calculated from double-reciprocal plots of AlPase activity against substrate concentration (Tab. 2). The *Kₐ* for p-NPP was found to be 0.5 mM. Similar values have been described for *Peridinium cinctum* (Kₐ = 0.45 mM; Carpene and Wynne 1986), *Pythococcus blakesleeanus* (Kₐ = 0.27 mM; Cohen 1985) and *Vibrio cholerae* (Kₐ = 0.22 mM; Roy et al. 1982). An inverse correlation between Vₜₐₜₜ and Kₐ for natural substrates was observed (Tab. 2). AlPase showed high affinity for PEP, NADP⁺, AMP, ADP and Ribose-5-phosphate. The *Kₐ* values for these natural substrates were 2.4, 4.0, 4.5, 5.8 and 6.1 mM, respectively. Other compounds such as fructose-1,6-bisphosphate, fructose-6-phosphate, glucose-6-phosphate and glucose-1-phosphate were poorly hydrolyzed; no effect on ATP, phosphocreatine and triphosphophate was detected, suggesting that these are not natural substrates for the enzyme. In cucumber roots nucleoside di- and triphosphates are hydrolyzed by the cucumber AlPase, whereas monophosphates are not (Yamaya and Matsumoto 1981). ATP is not hydrolyzed by AlPase from *Candida tropicalis* (Bedu et al. 1982) and *Vibrio cholerae* (Roy et al. 1982).

Enzyme activity was linear from 1.5 to 12 µg of the partially purified protein (Fig. 8A). As shown in Fig. 8B, the production of p-nitrophenol from p-NPP was linear during the first 6 min, suggesting that AlPase was not inhibited by the hydrolysis products. The AlPase could be stored at −10°C for at least 2–3 months without any loss of activity.

Phosphate inhibition

Phosphate inhibited the AlPase activity with 30% at 30 mM P, and 60% at 140 mM P (Fig. 9). The inhibition by P was competitive and Kₐ for P was 85 mM (data not shown).

Effects of metal ions and some anions

Table 3 summarizes the effect of various substances on the AlPase activity. The cations, Mg²⁺ and Co³⁺, had a weak inhibitory effect on the enzyme. Ni²⁺ increased the activity by 25% and Zn²⁺, Hg²⁺ and K⁺ had a strong inhibitory effect. Fluoride, ammonium and molybdate inhibited 20%, 30% and 80%, respectively.

In *Bacillus* sp. OK-1, AlPase activity is completely restored by the addition of cobalt ions, and partially by zinc ions (Nomoto et al. 1988) while the AlPase from *Blastocladia emersonii* does not require a divalent cation (Selitrennikoff and Sonneborn 1977). AlPase from *Pisum sativum* and *Cucumis sativus* is inhibited by potassium and calcium, respectively (Yamaya and Matsumoto 1981, Nougarde et al. 1983).

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References


