PARTIAL PURIFICATION AND CHARACTERIZATION OF A NONGLYCOPROTEIN ACID PHOSPHATASE FROM FIELD BEAN (*Lablab purpureus* L.)

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Affectionately Dedicated to

My Beloved Parents

and

Brother

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF AGRICULTURAL SCIENCES, BANGALORE

CERTIFICATE

This is to certify that the thesis entitled "**PARTIAL PURIFICATION AND CHARACTERIZATION OF A NONGLYCOPROTEIN ACID PHOSPHATASE FROM FIELD BEAN (Lablab purpureus L.)**" submitted in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE** in **AGRICULTURAL BIOCHEMISTRY** to the University of Agricultural Sciences, Bangalore, is a record of research work done by **Mr. ATMAKURI KRISHNAMOHAN**, under my supervision and guidance and that no part of the thesis has been submitted for the award of any degree, diploma, associateship, fellowship or other similar titles.

Bangalore
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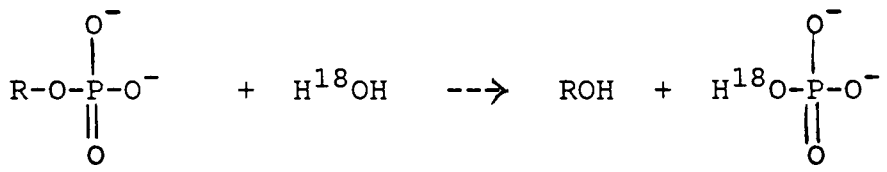
INTRODUCTION

I. INTRODUCTION

Phosphorus is one of the major nutrient elements required by plants for their normal growth and development. It is a major component of nucleic acids, cofactors like NAD^+, $NADP^+$, ATP and several other important biomolecules. It promotes root development and vigor of the plants, enhances nitrogen fixation, emergence of ears and grain formation and promotes crop maturation. Its deficiency affects root growth, crop maturity and protein synthesis.

Germinating seeds obtain phosphorus from phosphate reserves in the cotyledons by the activity of acid and alkaline phosphatases and phytases. Acid phosphatases **(EC.3.1.3.2)** are specific in hydrolyzing only the O-substituted monoesters of orthophosphoric acid while alkaline phosphatases also hydrolyze S-substituted monoesters. They are maximally active in the acidic pH range, catalyze the hydrolysis of a variety of phosphate esters and are found widely distributed in nature (Biswas and Cundiff, 1991).

Acid phosphatase catalyzes an apparent transition state displacement and a P-O cleavage according to the following equation.



In plants, acid phosphatase activity in seeds often increases greatly during germination (Rossi et al., 1981; Bhargava and Sachar, 1987 ; Pasqualini et al., 1992). Acid phosphatases with phosphoprotein phosphatase activity also have been detected (Chung and Polya, 1992; Gellatly et al., 1993) indicating their involvement in metabolic regulation by mechanisms involving phosphorylation and dephosphorylation.

The activity of acid phosphatase in plants (Toshiaki and Sakai, 1991; Guthrie et al., 1991) usually increases under phosphate deficient conditions indicating the occurrence of scavenging of various phosphomonoesters.

The role of acid phosphatase in plants during normal development, abiotic and biotic stress seems to be an important one.

However, the physiological role of acid phosphatase remains uncertain even after recent progress in isolation of the genes encoding for the enzyme. Hence the purification and characterization of an enzyme are the essential pre-

requisites to define the function or the physiological significance of an enzyme.

The purification and characterization of acid phosphatase from Lablab purpureus L., a legume, commonly referred to as field bean was undertaken with the following objectives:

1) Isolation and partial purification of acid phosphatase from the cotyledons of germinating Lablab purpureus seeds, and

2) Characterization of the acid phosphatase so purified.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The hydrolysis of phosphate monoesters in biological systems is a crucially important process linked to energy metabolism, metabolic regulation and to a wide variety of cellular signal transduction pathways. Despite the apparent simplicity of the problem, biological hydrolysis of phosphate monoesters is accomplished by a diverse group of enzymes, the phosphatases, that differ in very significant ways.

Acid phosphatases **(EC.3.1.3.2)** are a group of hydrolases that nonspecifically catalyze the hydrolysis of a variety of orthophosphoric monoester compounds in an acidic environment and are widely distributed in nature (Dixon and Webb, 1979). They occur in small quantities as isoforms and are unstable in very dilute solutions (Shinshi and Kato, 1979; Miernyk, 1992), making their isolation quite difficult.

Research on acid phosphatases is mainly focused on the clinical aspects and its significance, after its isolation from several animal sources such as beef spleen (Campbell and Zerner, 1973), mammalian reproductive organs (Schlosnagle et al., 1974), bovine brain (Ahmed et al., 1990) and frog liver (Panara et al., 1990a). They have also

been isolated from several micro-organisms like Rhodotorula rubra (Wieslaw et al., 1977), plasmodia of Physarum polycephalum (Kaneko and Katoh, 1990) and Nocardia braziliensis (Colon et al., 1992).

Thrust on plant acid phosphatases research in recent years resulted in purification and characterization of the enzyme from several plant sources such as tobacco (Veki and Sato, 1971), pea (Murray and Collier, 1977), rice (Yamagata et al., 1980), maize (Rossi et al., 1981), papaya (Carreno and Chan, 1982), blackgram (Tamura et al., 1982a), peanut (Basha, 1984), rye gram (Ferens and Morawiecka, 1985), sunflower (Park and Van Etten, 1986), red kidney bean (Beck et al., 1986), soybean (Okuda et al., 1987), cotton (Bhargava and Sachar, 1987), winged bean (Kamala Devi and Madhusudanan, 1989), mung bean (De-Kundu and Banerjee, 1990), black mustard (Duff et al., 1991), Vigna sinensis (Biswas and Cundiff, 1991), potato (Polya and Wettenhall, 1992), Japanese radish (Yashimoto et al., 1992) and field bean (Vinayaka, 1992 and Ramesh, 1994).

1. ACTIVITY DURING GERMINATION:

Acid phosphatase activity in seeds often increases greatly during germination indicating a possible role in phosphate mobilization.

A gradual increase in the acid phosphatase (AcPase) activity was observed in maize scutellum from 0 to 96 h germination (Rossi et al., 1981; Teno et al., 1987). In Vigna mungo seedlings a similar kind of increase in enzyme activity was observed, also indicating that the attachment of the axis organs to the seeds is not required for the increase in the enzyme activity (Tamura et al., 1982a; Haraguchi et al., 1990).

A marked incorporation of ^{14}C and ^{3}H radioactivity in the acid insoluble fraction after 24 h of germination of soybean seeds indicating the de novo synthesis of protein and RNA, was further ascertained by the significant inhibition of enzyme activity in embryo by cycloheximide in germinating soybean seeds (Okuda et al., 1987) and in cotton seedlings (Bhargava and Sachar, 1987). A gradual increase in AcPase activity in aleurone layers was observed after incubation of barley seeds in water at 25°C for 3 days (Gabard and Jones, 1986).

A 16-fold rise in the AcPase activity after 72 h of germination was observed in cotton seedlings (Bhargava and Sachar, 1987) while a 6.5 fold increase in specific activity after 72 h germination was observed in cotyledons of Lablab purpureus (Vinayaka, 1992).

A continuous increase of enzyme activity upto 9 days of germination in soybean cotyledons (Kaneko et al., 1990) and winged bean seeds (Kamala Devi and Madhusudanan, 1989) was observed. AcPase isolated from barley coleoptiles showed an increase in its specific activity between days 4 and 7 (Pasqualini et al., 1992).

2. DETECTION AND MEASUREMENT OF ACTIVITY:

The activity of acid phosphatase is generally measured using para-nitrophenyl phosphate (pNPP) as the substrate. The activity is determined by measuring the rate of formation of p-nitrophenol from enzyme catalyzed hydrolysis of pNPP substrate (Parish, 1974). When other phosphate compounds are used as substrates, the liberated inorganic phosphate is measured (Ames, 1966).

Enzyme activity in the gels after polyacrylamide gel electrophoresis is detected by incubating the gels in an enzyme staining solution containing 0.1% α-naphthyl phosphate, 1mM $MgCl_2$ and 1mg per ml Fast Red TR in 200 mM citrate buffer, pH 4.8, till zones exhibiting enzyme activity appear as coloured bands. This method of staining is a modification of Barka (1961). Even Fast Blue RR can be used in place of Fast Red TR.

3. PURIFICATION:

Several of the conventional techniques viz., alcohol precipitation, ammonium sulphate precipitation, ion-exchange chromatography, gel filtration and PAGE have been used in several convenient combinations for purification of acid phosphatases.

The above techniques have been used to purify AcPases from several plant sources such as sweet potato tubers (Sugiura et al., 1981), peanut (Basha, 1984), sunflower seeds (Park and Van Etten, 1986), cotton seedlings (Bharghava and Sachar, 1987) chickpea seeds (Angosto et al., 1988), soybean seeds (Ullah and Gibson, 1988), barley roots (Panara et al., 1990b), wheat germ (Parvin and Van Etten, 1991a), french bean (Smith et al., 1992), rice (Chen et al., 1992), maize endosperm (Miernyk, 1992), poppy seeds (Chung and Polya, 1992), barley coleoptiles (Pasqualini et al., 1992), soybean root nodules (Sarath et al., 1993) and field bean (Ramesh, 1994).

Affinity chromatography has been performed to purify glycoprotein acid phosphatases. Con A-Sepharose was used to purify AcPase from barley coleoptiles (Pasqualini et al., 1992), maize endosperm cultures (Miernyk , 1992), Japanese radish (Yashimoto et al., 1992) soybean suspension cultures (LeBansky et al., 1992) soybean root nodules (Sarath et al.,

1993) and field bean cotyledons (Vinayaka, 1992), while Con A-Agarose was used to purify glycoprotein acid phosphatases from cotton seedlings (Bhargava and Sachar, 1987) and wheat seeds (Saluja et al., 1989).

4. PHYSICAL PROPERTIES:

AcPases isolated from several sources exist as isoforms.Two isoforms were detected by PAGE in cotton seedlings (Bhargava and Sachar, 1987), in seedling axes of Vigna radiata (De-Kundu and Banerjee, 1990), in phosphorus deficient wheat plants (Guthrie et al., 1991) and in cytoplasmic extract of barley roots, whereas, the cell bound fraction of barley roots had three isoforms (Panara et al., 1990b). Three isoforms were detected by non-denaturing PAGE in soybean root nodules (Sarath et al., 1993) and in 48 h germinated cotyledons of Lablab purpureus (Vinayaka, 1992) while four isoforms were detected in Vigna mungo seeds on performing ion-exchange chromatography and PAGE (Tamura et al., 1982b) and in preparation of 96 h germinating seeds of Vigna sinensis (Biswas and Cundiff, 1991).

Five isoforms were detected under non-denaturing PAGE conditions in axes of 72 h and 96 h germinated Lablab purpureus seeds (Ramesh, 1994). Six isoforms, AP-Ia_1, Ia_2, Ib_1, Ib_2, IIa and IIb were detected on performing column chromatography followed by gel electrophoresis in Vigna

mungo cotyledons (Haraguchi et al., 1990). Nine isoforms were detected in aleurone layers of barley (Gabard and Jones, 1986) while 10 isoforms were detected in rice seedlings based on separation by PAGE (Chen et al., 1992).

The endosperm of date palm (Phoenix dactylifera) seeds had six isoforms of AcPases while the haustorium from the same seeds contained as many as fourteen isoforms detected by Isoelectric focusing (Chandrashekar and DeMason, 1989).

5. MOLECULAR WEIGHT:

The molecular weights (M_rs) of various plant acid phosphatases show a wide variation ranging from 21,500 as in haustorium of date palm seeds (Chandrashekar and DeMason, 1989) to 2,40,000 as observed for the peanut enzyme (Basha, 1984).

The AcPases from plants exist both as monomeric and multimeric forms. Some of the isolated AcPases which are monomeric in nature were of M_r 2,10,000 from Shii-take, a fruit body of Lentinus edodes (Takako and Endo, 1987), of M_r 1,50,000 from germinating seeds of Vigna sinensis (Biswas and Cundiff, 1991), of M_r 1,85,000 from embryoless half seeds of wheat (Saluja et al., 1989) and of M_r 1,01,000 and 1,18,000, of the two isoforms purified from seedling axes of Vigna radiata (De-Kundu and Banerjee, 1990). The AcPases of

approximate M_r of 50kDa isolated from `P' deficient wheat plants (Guthrie et al., 1991), cotyledons of Vigna mungo (Haraguchi et al., 1990) and cotyledons of germinating soybean seeds (Ullah and Gibson, 1988) were also monomeric.

Some of the multimeric forms of AcPases were, the enzyme purified from peanut seed of M_r 2,40,000 which had six identical subunits each of M_r 42,500 (Basha, 1984); the enzyme from plasma membranes of peanut cotyledons which was heterodimer of M_r 46.7 and 50.1 kDa (Basboa et al., 1987) and the enzyme purified from the soybean suspension cultures which was a homodimer of subunit of M_r 58,000. The glycoprotein enzyme from cotyledons of field bean was a heterodimer of M_r 1,27,000, with subunits of M_r 64,000 and 63,000 (Vinayaka, 1992).

6. ISOELECTRIC POINT:

The isoelectric points (pIs) of a few plant acid phosphatases have been reported and it varies from 3.6 for an isoenzyme from endosperm of date palm seeds (Chandrashekar and DeMason, 1989) to 6.7 obtained by chromatofocusing in cotyledons of germinating soybean seeds (Ullah and Gibson, 1988). The Mn-III containing acid phosphatase purified from sweet potato tubers (Sugiura et al., 1981) had a pI of 5.18 while the one purified from peanut seed had a pI of 5.6 (Basha, 1984). The AcPases

purified from plump and shriveled seeds of triticale had a pI of 5.9 (Ching et al., 1987). The four isoforms F_1, F_2, F_3 and F_4 isolated from rice bran exhibited pIs of 5.1, 5.1, 5.6 and 5.9, respectively (Hayakawa et al., 1989) while the six isoforms of endosperms and fourteen isoforms of haustorium from date palm seeds exhibited a pI range of 3.6 to 4.3 and 5.3 to 6.8 respectively (Chandrashekar and DeMason, 1989). The purple acid phosphatase purified from soybean suspension cultures exhibited a pI of 5.0 (LeBansky, 1992). The major cytoplasmic isoenzyme purified from the barley root acid phosphatase exhibited a pI of 6.28 (Panara et al., 1990b). The Lablab purpureus cotyledonary glycoprotein enzyme showed a pI of 5.4 (Vinayaka, 1992).

7. CHEMICAL NATURE OF ACID PHOSPHATASE:

Some of the isolated and purified AcPases have been found to be glycoproteins. The AcPases purified from germinating soybean seeds (Ullah and Gibson, 1988), sunflower seeds (Park and Van Etten, 1986), rice bran (Hayakawa et al., 1989), embryoless half seeds of wheat (Saluja et al., 1989) Vigna mungo seeds (Haraguchi et al., 1990) cotyledons of germinating soybean seeds (Kaneko et al., 1990), black mustard cell suspensions (Duff et al., 1991), soybean suspension cultures (LeBansky et al., 1992), field bean cotyledons (Vinayaka, 1992) were all glycoproteins.

The AcPase purified from 96 h germinated maize scutellum was shown to have 16 per cent neutral sugars (Rossi et al., 1981), and the enzyme from plump and shriveled seeds of triticale contained 12 per cent carbohydrate (Ching et al., 1987). The enzyme purified from plasma membrane of peanut cotyledon had 50 per cent carbohydrate (Basboa et al., 1987). The purified enzyme from maize endosperm cultures contained approximately 6 per cent neutral sugars, primarily, mannose, fucose and xylose (Miernyk , 1992).

However, not all acid phosphatases are glycoproteins. The enzymes from Vigna radiata (De-Kundu and Banerjee, 1990), Vigna sinensis (Biswas and Cundiff, 1991), peanut (Basha, 1984), rice seedlings (Chen et al., 1992) and field bean (Ramesh, 1994) were all found to be nonglycoprotein in nature.

The amino acid analysis of AcPases from soybean (Fujimoto et al., 1977), sunflower (Park and Van Etten, 1986), red kidney bean (Jennifer et al., 1986), tomato (Tanaka et al., 1990), black mustard cell suspension cultures (Duff et al., 1991) and potato tuber (Polya and Wettenhall, 1992) have also been reported.

8. SPECIFICITY:

The AcPases have been shown to hydrolyze a wide variety of compounds like fructose-1,6-bisphosphate and $NADP^+$ (Sugiura et al., 1981), ATP and ADP (Tamura et al., 1982b), ATP and pyrophosphate (Ferens and Morawiecka, 1985), glucose-6-phosphate, fructose-1,6-bisphosphate, ribose-5-phosphate and pyrophosphate (Angosto et al., 1988), pyrophosphate, ATP and phosphoenolpyruvate (Kaneko et al., 1990), ATP, tripolyphosphate and tetra sodium pyrophosphate (Guthrie et al., 1991 & Van Etten and Parvin, 1991b), phosphoenolpyruvate, ATP and ADP (Biswas and Cundiff, 1991; Pasqualini, et al., 1992), 2'-AMP, 3'-AMP, 5'-AMP, O-phospho- L-serine, O-phospho-L-threonine and O-phospho-L-tyrosine (Ching and Polya, 1992), O-phospho-L-serine and O-phospho-L- tyrosine (Gellatly et al., 1993) and sodium pyrophosphate, sodium phytate and ADP (Ramesh, 1994) indicating that they have a wide range of substrate specificity.

9. KINETIC PROPERTIES:

The AcPases isolated so far from various sources have been shown to follow Michaelis-Menten kinetics. The Km for pNPP varies from as low as 0.2μM for the peanut enzyme (Basha, 1984) to as high as 10 mM for the enzyme isolated from the plasma membrane of peanut cotyledons (Basboa et

al., 1987) The purple acid phosphatase from soybean suspension cultures had a Km of 0.3 mM for pNPP as substrate (Le Bansky et al., 1992), while the enzyme from barley coleoptiles exhibited a Km of 0.10 mM for the same substrate (Pasqualini et al., 1992). The AcPases from tomato leaves showed a Km of 2.9 mM for pNPP (Tanaka et al., 1990), while it was 5 mM and 1.92 mM for the enzyme purified from axes and cotyledons of Lablab purpureus respectively (Ramesh, 1994 & Vinayaka 1992). The AcPases purified from wheat germ exhibited a Km of 5.7±0.95 and 1.9±0.09 mM for pNPP at pH 5.0 and pH 7.0 indicating that the pH also influences the Km for a substrate (Van Etten and Parvin, 1991b).

10. TEMPERATURE OPTIMUM AND THERMAL STABILITY:

The AcPases also exhibit a wide temperature optima. The AcPase purified from axes of germinating cotyledons of field bean (Ramesh, 1994), papaya (Carreno and Chen, 1982) and cotton seedlings (Bhargava and Sachar, 1987) exhibited an optimal temperature of 37°C, while the enzyme from black mustard cell suspension cultures had a temperature optimum of 67°C (Duff et al., 1991). The AcPases isolated from Shii-take (Takako and Endo, 1987), germinating soybean seeds (Ullah and Gibson, 1988) and the two isoforms of Vigna radiata seedlings (De-Kundu and Banerjee, 1990) had an optimum temperature of 60°C. Of the multiple forms of acid phosphatases isolated from germinating seeds of Vigna

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sinensis, the temperature optimum for AP-I and IV was 55°C while it was 60°C and 65°C for AP-II and III, respectively (Biswas and Cundiff, 1991). The AcPases purified from Lablab purpureus cotyledons (Vinayaka, 1992) and maize endosperm cultures (Miernyk, 1992) exhibited an optimum of 48° and 50°C, respectively.

The AcPases vary in their thermal stability too. The enzymes from axes and cotyledons of germinating soybeans separately were stable even for a period of one year at -20°C when stored at pH 5.8. Only 10 per cent of the activity was retained after 60 minutes at 60°C when they were monitored separately. However, when both enzymes were incubated together at pH 4.0 and 5.0 for 60 minutes at 60°C, 60 and 46 per cent of their activity was lost, respectively (Kaneko et al., 1990). The maize enzyme's activity was rapidly and irreversibly lost at 60°C with a half life of 6 minutes. The purified enzyme was, however, relatively stable at 50°C with a half life of 40 minutes (Miernyk, 1992). The AcPase purified from germinating cotyledons of Lablab purpureus lost 21-27 per cent of activity in 10 minutes and 43 to 64 per cent in 60 minutes when incubated at 45°, 55°, 60° and 65° C (Vinayaka, 1992).

The Arrhenius plots for the AcPases from several sources were constructed and the activation energy reported (Tamura et al., 1982b; De-Kundu and Banerjee, 1990; Duff et

al., 1991; Vinayaka, 1992; Ramesh, 1994). The activation energy range from 1.54 kcal mol^{-1} to 13.5 kcal mol^{-1}.

11. pH OPTIMUM:

The AcPases show maximum activity in the acidic pH range. An optimum of pH 5.0 was exhibited by all the 4 AcPase isoforms isolated from germinating Vigna sinensis seeds (Biswas and Cundiff, 1991), AcPase of rice seedlings (Chen et al., 1982), a AcPase exhibiting phosphoprotein phosphatase activity from poppy seeds (Chung and Polya, 1992), the enzyme purified from germinating soybean seeds (Ullah and Gibson, 1988) and the enzyme isolated from the adaxial region of cotyledons of Lupinus luteus (Rehana and DeMason, 1992). A pH optimum of 4.5 was shown by AcPases from tomato leaves (Tanaka et al., 1990) and abaxial regions of cotyledons of Lupinus luteus (Rehana and DeMason, 1992). The AcPases of wheat germ (Parvin and Van Etten, 1991a) and the enzyme from plasma membrane of peanut cotyledons (Basboa et al., 1987) showed pH optima of 4.7. The optimal pH for AcPase from the scutella of germinating maize seeds was 6.7 (Rossi et al., 1981). Each of the two acid phosphatases from Euphorbia lathyria latex had two pH optima: $AP\text{-}I_1$ at 5.0 and 6.8 and $AP\text{-}I_2$ at 5.8 and 7.5 (Lynn and Clevette-Radford, 1987).

12. EFFECT OF METAL IONS AND OTHER REAGENTS

A. METAL IONS:

Metal ions have been shown to either inhibit or activate the acid phosphatases from different sources. Mg^{2+} and Mn^{2+} have been shown to activate acid phosphatases purified from potato tuber (Gellatly et al., 1993), Japanese radish (Yashimoto et al., 1992), rice seedlings (Chen et al., 1992) and barley coleoptiles (Pasqualini et al., 1992).

Ni^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} have been shown to activate the AcPases purified from Japanese radish (Yashimoto et al., 1992) while Zn^{2+} and Cu^{2+} have been shown to inhibit the poppy seed AcPase drastically (Chung and Polya, 1992). Hg^{2+}, Cu^{2+}, MoO_4^{2-} inhibit strongly the acid phosphatase from barley coleoptiles while Rb^{+}, Ca^{2+}, Ni^{2+} and Zn^{2+} activated the enzyme activity (Pasqualini, et al., 1992). Ca^{2+}, Fe^{3+}, Hg^{2+}, Mo^{6+}, Zn^{2+} and Cu^{2+} inhibit the enzyme isolated from germinating seeds of Vigna sinensis (Biswas and Cundiff, 1991). Zn^{2+} with and without EDTA, Cu^{2+}, Hg^{2+}, Ag^{+} and arsenate inhibited both AP-I and AP-II from seedling axis of Vigna radiata while Ca^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} activated both the isoforms (De-Kundu and Banerjee, 1990).

Mg^{2+}, Ca^{2+}, Hg^{2+}, Fe^{2+} and Zn^{2+} inhibited the AcPase

purified from germinating chickpea seeds (Angosto et al., 1988) while Hg^{2+} marginally activated the enzyme activity obtained from the pollen of Brassica campestris and Lilium regale (Strother et al., 1985).

B. GROUP SPECIFIC REAGENTS:

The effect of several group specific reagents on acid phosphatases have been reported.

The loss of enzyme activity in the presence of iodoacetamide and p-chloromercuribenzoate (PCMB) was observed in rye germ acid phosphatase suggesting a role for free sulfhydryl groups in supporting the active conformation or the catalytic process (Ferens and Morawiecka, 1985). The acid phosphatase activity was unaffected by PCMB in peanut (Basha, 1984).

A 10 mM concentration of β-mercaptoethanol and dithiothreitol separately, and in combination, brought about a 2.5 to 3.0 fold activation of AcPase, thereby indicating the requirement of free -SH groups for the catalytic efficiency of the enzyme purified from the embryoless half seeds of wheat (Saluja et al., 1989). All the four isoforms of the enzyme isolated from Vigna sinensis were inhibited by 1mM PCMB noncompetitively (Biswas and Cundiff, 1991). AP-I and AP-II from Vigna radiata were not

significantly influenced by β-mercaptoethanol but PCMB caused some inhibition of AP-I (De-Kundu and Banerjee, 1990). The cotyledonary enzyme from field bean was not affected by PCMB and 2-mercaptoethanol, but was inhibited by iodoacetate (Vinayaka, 1992) while the one from axes was not affected by PCMB and iodoacetate, but was marginally activated by 2-mercaptoethanol (Ramesh, 1994).

C. OTHER REAGENTS:

Arsenate was found to inhibit the acid phosphatase purified from germinating chickpea seeds (Angosto et al., 1988). Vanadate and sodium fluoride were observed to inhibit the enzyme activity isolated from potato tubers (Gellatly et al., 1993), rice seedlings (Chen et al., 1992) and poppy seeds (Chung and Polya, 1992). Inorganic phosphate, sodium fluoride and pyrophosphate were found to inactivate the enzyme from black mustard-cell suspension cultures (Duff et al., 1991) while Triton-X-100, ethanol, acetone, formaldehyde and tartrate activated the enzyme of barley coleoptiles (Pasqualini et al., 1992). EDTA had no effect on AcPase of wheat germ (Parvin and Van Etten, 1991a) while it increased the activity of acid phosphatases from barley coleoptiles (Pasqualini et al., 1992) and from pollen of Brassica campestris and Lilium regale (Strother et al., 1985). Tween-80 activated both the isoforms of Vigna radiata seedlings (De-Kundu and Banerjee, 1990). The field

bean cotyledonary and axes AcPases were unaffected by Triton-X-100 but inhibited by SDS (Vinayaka, 1992; Ramesh, 1994). N-ethyl maleimide and 1,10-phenanthroline enhanced the activity of the AcPase from seedcoats of developing pea seeds (Murray, 1980).

13. PHYSIOLOGICAL ROLE:

According to Ferte et al., (1993), AcPases are present in cell walls, vacuoles and laticifers, especially, in the lumen of provacuolar and vacuolar apparatus of non articulated laticifers (Giordani et al.,1986), where they may be involved in turn over of cytoplasmic components. It is also thought to be involved in trapping and transporting phosphate esters from the apoplastic to the cytoplasmic compartment in the plant cells. The hydrolysis of phosphoamino acids like O-phospho-L-tyrosine and O-phospho-L- serine (Panara et al., 1990b) by AcPases suggests a possible role for them in signal transduction where proteins usually get reversibly phosphorylated. The regulation of several important enzymes and proteins in several biochemical pathways are controlled by phosphorylation and dephosphorylation mechanism eg., in glycogen phosphorylase, histones and cell cycle proteins especially at the serine, threonine and tyrosine residues. Thus the phosphoprotein phosphatases in combination with protein kinases may regulate several biochemical pathways in

response to internal and external stimuli (Biswas and Cundiff, 1991).

According to LeBansky et al., (1992), although the role of vacuolar AcPase is unclear, the secreted AcPase may be used by plants to scavenge phosphate from organic sources under phosphate limiting conditions.

Ullah and Gibson (1988) suggest that the AcPase isolated from soybean cotyledons mobilizes phosphate from phytic acid during germination.

According to Yoshimoto et al., (1992), the AcPases also hydrolyze a group of taste substances in foods like sodium-5'-inosinate (5'-IMP) and sodium-5'-guanylate to phosphate and nucleosides.

Chandrashekar and DeMason (1988) concluded from the differential activity of AcPases of the endosperm and haustorium of date palm seeds that, phosphate metabolism in the endosperm is independent of that in the haustorium and that the AcPases for endosperm phosphate mobilization are not secreted by the embryo or the haustorium, but instead are stored in the endosperm.

Biswas and Cundiff (1991) conclude that the

physiological significance of different forms of AcPase in the germinating seeds is uncertain. However, its activity needs to be increased to recycle phosphate in proliferating embryonic cells. The cell tries to achieve this goal by increasing the enzyme activity and by having isozymes with different affinities for substrates under the conditions existing within the cell. There is still speculation about the role of each isozyme having specific physiological influence on cellular development.

14. MECHANISM OF ACTION:

Very little information is known regarding the mechanism of action of acid phosphatase even after extensive work has been done on this phenomenon. However a few suggestions have been made based on studies using group specific reagents.

Five isoforms of acid phosphatase isolated from the lattices of three members of the Euphorbiaceae family have been grouped into various classes by Lynn and Clevette-Radford (1987) based on amino acid residues found at the active site of these enzymes.

Acid phosphatases $AP\text{-}1_1$ and $AP\text{-}1_2$ isolated from <u>Euphorbia</u> <u>lathyria</u> were inhibited by phenylmethyl sulphonyl fluoride (PMSF), PCMB and diethyl pyrocarbonate (DEPC)

indicating that they have serine, cysteine and histidine at their active sites while the two isoforms AP-d_1 and AP-d_2 were inhibited by PCMB alone indicating that cysteine could be the active site ligand.

The barley root acid phosphatase was inactivated by DEPC, indicating the involvement of histidine in catalysis (Panara et al., 1990b). Kaneko and Katoh (1990) have suggested a duoble displacement mechanism for a transphosphorylation reaction catalysed by acid phosphatase from Physarum polycephalum KH 25.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

MATERIAL

A: SEED SAMPLES:

Lablab purpureus L.(Dolichos lablab L.), variety, Hebbal Avare-3 seeds were obtained from the National Seed Project, University of Agricultural Sciences, GKVK Campus, Bangalore and also from the Agricultural Research Station, Kathalagere, Shimoga.

B: CHEMICALS:

Phenylmethylsulphonylfluoride (PMSF), α-naphthyl phosphate, sodium pyrophosphate, 2-mercaptoethanol, Coomassie Brilliant Blue-G 250, Fast Red TR and protein molecular weight markers were obtained from Sigma Chemical Company, USA. TEMED was obtained from Koch-Light Laboratories Ltd., England.

DEAE-Sephadex A-50, Sephadex G-100 and Sepharose-4B and Ampholine (pH 3-10) were products of Pharmacia, Sweden. All other chemicals used were of the best reagent grade available.

PROTEIN ESTIMATION:

Protein was estimated by the modified technique of Bradford (Spector, 1978).

REAGENTS FOR MODIFIED BRADFORD'S METHOD:

60 mg of Coomassie Brilliant Blue G-250 were dissolved in 1 liter of 3 per cent perchloric acid and then filtered. Absorbance at 415 nm showed 1.3. The reagent was stable indefinitely.

STANDARD ALBUMIN SOLUTION:

A stock solution of BSA of 10 mg per ml concentration was prepared and diluted 1:100 to obtain a working standard containing a concentration of 100 μg BSA per ml.

STANDARD CURVE FOR PROTEIN:

Aliquots of BSA containing 10 to 50 μg BSA were pipetted out into a series of test tubes and the volume was made upto 1.5 ml with distilled water. Then 1.5 ml of Coomassie Brilliant Blue G-250 solution was added to each tube and the absorbance of the complex was read at 595 nm immediately. The absorbance was plotted against the

concentration levels to obtain the standard curve. Samples were appropriately diluted to estimate the protein content.

ACID PHOSPHATASE ASSAY:

The enzyme activity was measured using p-nitrophenyl phosphate (pNPP) as the substrate and estimating the p-nitrophenol released (Parish, 1974). The enzyme was assayed in a reaction mixture which contained 0.4 ml of 12.5 mM pNPP (dissolved in distilled water) 0.5 ml of 200 mM citrate buffer, pH 4.8 and 0.1 ml of appropriately diluted enzyme in a total of 1.0 ml. The assay was performed at 37°C with an incubation time of 5 minutes. The reaction was terminated by the addition of 2.0 ml of 0.2 M NaOH. The liberated p-nitrophenol was measured by reading the absorbance of the assay mixture at 405 nm.

STANDARD CURVE FOR p-NITROPHENOL:

A: Aliquots of standard p-nitrophenol (100 nmoles/ml) were pipetted out into a series of test tubes in the concentration range of 10 to 100 nmoles and the volume made upto 1 ml with distilled water. To each tube 2 ml of 200 mM NaOH was added and the yellow colour which developed soon after was read against a suitable blank at 405 nm. The absorbance was plotted against the different concentrations to obtain the standard curve. The activity of enzyme was

expressed in nkatals and 1 katal is defined as the amount of enzyme that hydrolyzes 1 mole of pNPP in one second, under the given assay conditions.

B: When other phosphate substrates were used, the activity of AcPase was assayed by measuring the amount of inorganic phosphate released, by the method of Ames (1966). The following reagents were used for phosphate estimation.

1. 10 per cent solution of ascorbic acid in water.
2. 0.42 per cent solution of ammonium molydate in 1 N sulfuric acid.

Reagents 1 and 2 were mixed in the proportion 1:6 and placed in ice. The two solutions were mixed just before use (Reagent A).

STANDARD PHOSPHATE SOLUTION:

A solution of sodium dihydrogen orthophosphate containing 31.2 mg in 100 ml of the solution was prepared. This solution was diluted 50 fold to get a standard solution containing 40 nmoles of inorganic phosphate per ml.

Aliquots of the standard phosphate solution containing 4 to 24 nmoles of inorganic phosphate were pipetted out into a series of test tubes and the volume was made up to 0.6 ml

with distilled water. 1.4 ml of Reagent A was added to each tube. The tubes were then incubated at 45°C for 20 minutes. The absorbance of the solutions were recorded at 820 nm.

DETERMINATION OF OPTIMUM pH, OPTIMUM TEMPERATURE AND THERMAL STABILITY OF CRUDE ENZYME EXTRACT:

Three days germinated seedlings were used for determination of optimum pH, optimum temperature and thermal stability. Cotyledons (5 g) were extracted using 15 ml of cold 100 mM citrate buffer, pH 4.6 containing 1 mM PMSF, for 30 minutes in the cold and then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was assayed for AcPase at the following pH values: pH 3.0, 3.4, 3.8, 4.0, 4.4, 4.6, 4.8, 5.0, 5.4, 5.8, 6.0, 6.4, 6.8, 7.0, 7.2 and 7.4 using pNPP as substrate to determine the optimum pH. The crude enzyme extract was assayed by the standard procedure as described earlier at the following temperatures: room temperature (26°C), 30°, 37°, 45°, 48°, 55°, 60°, 65°, 70° and 75°C to determine the optimum temperature. The thermal stability of the crude enzyme extract was tested at the following temperatures: 30°, 37°, 45°, 55°, 60° and 65°C. 1.5 ml of the crude enzyme extract was incubated at each temperature. 0.1 ml of the incubated enzyme extract was withdrawn into test tubes containing 0.4 ml of 100 mM citrate buffer, pH 4.6 and kept in ice. The enzyme was withdrawn at 10, 20, 30, 45 and 60 minutes from

the time of incubation. These aliquots were assayed for enzyme activity by the standard procedure.

1. ACTIVITY DURING GERMINATION:

The seeds were surface sterilized for 2-3 minutes with 0.1 per cent mercuric chloride and then washed extensively with water to remove the mercuric chloride. They were then germinated in the dark at room temperature for different periods of time ranging from 24 h to 120 h.

Crude enzyme extracts were obtained by homogenizing 5 g of cotyledons in 15 ml of 100 mM cold citrate buffer, pH 4.6 containing 1 mM PMSF and stirring for 30 minutes at $4^{o}C$. The extracts were centrifuged at 10,000 rpm for 25 minutes at $4^{o}C$ and the supernatants were used as the crude enzyme extracts. Enzyme activity and protein content in the crude extracts were measured by the methods described earlier. In case of resting seeds, the enzyme extract was prepared using 2.5 g of seed meal and 15 ml of 100 mM cold citrate buffer, pH 4.6 containing 1 mM PMSF.

ELECTROPHORESIS:

Disc gel electrophoresis of the proteins in the crude enzyme extract was carried out by the method of Reisfeld _et al._, (1962) in 6.0 per cent gels. 100 μg protein for

protein detection, 25 μg for enzyme staining and 150 μg protein for glycoprotein staining was electrophoresed at a current of 4 mA per tube. The tracking dye for the cathodic run (pH 4.5) was methyl green. Gels were stained for protein in Coomassie Brilliant Blue overnight at room temperature. The gels were destained using ethanol-water-formaldehyde (250:750:10; Burk et al., 1983).

For location of enzyme activity, the gels were placed in 1 M citrate buffer, pH 4.6 for 15 minutes. This was followed by incubation of the gels in the staining solution containing 0.1 per cent α-naphthyl phosphate, 1mg per ml Fast Red TR and 1mM $MgCl_2$ in 200 mM citrate buffer, pH 4.6. When the bands developed, the gels were transferred to 7.5 per cent acetic acid and stored.

Gels were stained for glycoproteins by the periodic acid-Schiff (PAS) reaction (Fairbanks et al., 1971). Gels were fixed in 7.5 per cent acetic acid for 1 h at room temperature and then transferred to 0.4 per cent periodic acid in the cold for 45 minutes. The gels were then washed with 7.5 per cent acetic acid in the cold overnight to remove periodic acid, then placed in Schiff's reagent and left in the cold for 12 to 20 h. The gels were rinsed in a the mixture of 50 ml 1 N HCl, 5 g potassium metabisulphite and 150 ml of distilled water which was prepared fresh and stored in the same solution.

2. PURIFICATION OF ACID PHOSPHATASE:

30 g of cotyledons from field bean seeds germinated for 120 h were homogenized in 90 ml of cold 100 mM Tris-HCl buffer, pH 8.0, containing 1 mM PMSF and kept stirring in the cold for 30 minutes. The extract was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was taken to 35 per cent saturation with ammonium sulphate. The supernatant after 35 per cent ammonium sulphate saturation was again taken to 65 per cent saturation with ammonium sulphate. The 35-65 per cent ammonium sulphate saturation pellet was dissolved in a minimum volume of extraction buffer and dialyzed against 100 mM Tris-HCl buffer, pH 7.2 overnight.

A column (2.0 x 25 cm) of DEAE-Sephadex A-50 was equilibrated with 100 mM, Tris-HCl buffer, pH 7.2. Approximately 100 mg protein from the ammonium sulphate fraction was loaded on to the column and eluted with the same buffer. 2 ml fractions were collected and monitored for enzyme activity and protein content. The bound proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in the same buffer. The peak activity fractions were pooled, dialyzed overnight against 100 mM Tris-HCl buffer, pH 7.2 and subjected to ultrafiltration at 3 kg cm^{-2} pressure (using nitrogen gas) using a 10 kDa cutoff membrane. The

concentrated fraction was then loaded onto a Sephadex G-100 (1.0 x 28 cm) column pre-equilbrated with 100 mM Tris-HCl buffer, pH 7.2 and then eluted with the same buffer. 3 ml fractions were collected. The peak activity fractions were pooled and then concentrated in a dialysis bag placed in powdered sucrose. The concentrated solution was applied onto a Sepharose-4B (1.0 x 41 cm) column pre-equilibrated with the above mentioned buffer and eluted with the same buffer, 1ml fractions were collected. The fractions with high enzyme activity and low contaminating non-enzymatic protein (as visualized by PAGE) were pooled and used for characterization of the enzyme. Vertical slab gel electrophoresis of the protein in the individual peak fractions and pooled fractions was carried out by the method of Reisfeld et al., (1962) in 6.0 per cent gels. The gels were stained for enzyme activity as described earlier. Proteins were detected by silver staining using a modified method of Blum et al., (1987).

CHARACTERIZATION OF THE PARTIALLY PURIFIED Lablab purpureus L. COTYLEDONS ACID PHOSPHATASE:

4. EFFECT OF pH:

The following buffers in the pH range of 3.0 to 8.0 were used.

pH 3.0 to 6.2 - 200 mM citrate buffer.

pH 6.4 to 7.0 - 200 mM Tris-Maleate and

pH 7.2 to 8.0 - 200 mM Tris-HCl.

The reaction mixture contained 0.4 ml of 12.5 mM pNPP and 0.5 ml of the buffer of required pH. The reaction was initiated by the addition of 0.1 ml of appropriately diluted enzyme and the reaction was carried out by the standard procedure. A plot of enzyme activity versus pH was drawn to obtain the pH optima.

5. EFFECT OF TEMPERATURE:

The temperatures chosen for this study were 24°, 30°, 37°, 42°, 48°, 55°, 60°, 65° and 70°C. The standard reaction mixture (0.4 ml of 12.5 mM pNPP, 0.5 ml of 200 mM citrate buffer, pH 4.8) was incubated for 5 minutes with 0.1 ml of appropriately diluted enzyme at different temperatures. A plot of activity versus temperature was drawn to obtain the optimum temperature.

The thermal stability of acid phosphatase was tested at the following temperatures: 37°, 45°, 55°, 60° and 65°C. 1.5 ml of the appropriately diluted enzyme was incubated at each temperature. 0.1 ml of the incubated enzyme was with drawn into test tubes containing 0.4 ml of 100 mM citrate buffer, pH 4.8 and kept in ice. The enzyme was withdrawn at 10, 20,

30, 45 and 60 minutes from the time of incubation. The activity of the enzyme aliquots withdrawn was assayed by the standard procedure.

5. EFFECT OF SUBSTRATE CONCENTRATION:

pNPP in the concentration range of 1 mM to 35 mM prepared in distilled water was used to study the effect of different substrate concentration on AcPase activity. A velocity versus substrate concentration curve (right rectangular hyperbola curve) and the Lineweaver-Burk plot were drawn and the Km calculated from the Lineweaver-Burk's plot.

6. SUBSTRATE SPECIFICITY:

The following compounds were used at 10 mM concentration to study the substrate specificity of AcPase: D-glucose-6-phosphate, D-glucose-1-phosphate, D-fructose-1-phosphate barium salt, D-fructose-1,6-bisphosphate, α-naphthyl phosphate, pyrophosphate disodium salt, sodium phytate from rice, O-phospho-L-serine, O-phospho-L-tyrosine, sodium-ß-glycero phosphate, 5'-ATP, 5'-ADP, 5'-AMP, 5'-UMP, 5'-CMP and $NADP^+$.

The activity of the enzyme on these substrates was measured by estimating the liberated inorganic phosphate.

The assay mixture consisted of 0.3 ml substrate, 0.2 ml of 200 mM citrate buffer, pH 4.8 and 0.1 ml of appropriately diluted enzyme. This was incubated at 37°C for 10 minutes and the inorganic phosphate liberated was estimated as described earlier. The results were expressed as per cent relative activity considering the activity of the enzyme with pNPP as 100 per cent.

7. EFFECT OF METAL IONS AND VARIOUS REAGENTS:

The following metal ions were used at concentration of 1mM: Ag^{+} (silver nitrate), Zn^{2+} (zinc chloride), Cu^{2+} (copper sulphate), Hg^{2+} (mercuric chloride), Co^{2+} (cobalt chloride), Fe^{2+} (ferrous sulphate), Mg^{2+} (magnesium chloride), tungstate (sodium tungstate) and molydate (sodium molydate).

The following reagents were used at 1 mM concentrations: PCMB, iodoacetic acid, D (+)-tartaric acid, sodium fluoride and potassium cyanide.

Triton-X-100 (0.5 per cent), 2-mercaptoethanol (5 mM), sodium phosphate (dibasic)-25 mM and sodium dodecyl sulphate (0.1 per cent) were also used. The partially purified enzyme (67.5 μg) was pre-incubated with the required concentration of metal ions or reagents at room temperature for 10 minutes. The residual activity of the enzyme was

determined by the standard assay procedure. In the control, the enzyme was not pre-incubated with any metal ion or reagent. Results were expressed as per cent relative activity assuming the control to have 100 per cent activity.

8. MOLECULAR WEIGHT:

The molecular weight of nonglycoprotein AcPase was determined by gel filtration on Sepharose-4B as described in Sigma Technical Bulletin No.GF-3 which is a modification of the method of Andrews (1964).

A Sepharose-4B (1.8 x 72 cm) column was prepared in 50 mM Tris-HCl, pH 7.2, containing 100 mM KCl and equilibrated with the same buffer. Once the column was packed it was continuously eluted with the same buffer.

The void volume (V_o) of the column was determined by determining the elution volume of blue dextran. This was done by measuring spectrophotometrically ($A_{280\ nm}$) the volume of effluent collected from the point of application of blue dextran to the centre of the peak. Two mixtures of the following protein standards were run separately, on the column at a flow rate of approximately 20 ml per hour:

Mixture-I: Carbonic anhydrase (MW:29,000); alcohol dehydrogenase (MW:1,50,000) and thyroglobulin (MW:6,70,000).

Mixture-II: Bovine serum albumin (MW:66,000) and catalase (MW:2,32,000).

Elution of these standards was followed by reading absorbance of the fractions at 280 nm. The V_e for the protein standards and the enzyme protein were determined spectrophotometrically by measuring the volume of effluent collected from the point of sample application to the centre of the effluent peak.

A calibration curve of molecular weight versus V_e/V_o for the protein standard was plotted on semilog graph paper. The molecular weight of the AcPase was estimated from the calibration curve.

9. DETERMINATION OF ISOELECTRIC POINT (pI):

Isoelectric focusing was performed according to the method of Righetti (1983) in 7.5 per cent gel. The gel was polymerized with ammonium persulphate as catalyst and incorporating ampholyte (Pharmacia, pH range 3-10). 18 μg of the enzyme protein was applied on to the gel and run at 150 V for three hours at room temperature. The gel was then placed in 1 M citrate buffer, pH 4.8 for 10 minutes and then transferred into an AcPase staining solution containing 0.1 per cent α-naphthyl phosphate, 1 mM magnesium chloride and 1 mg per ml Fast Red TR in 200 mM citrate buffer, pH 4.8 and

incubated for 15 minutes at 37°C. The gel was then washed with 7.5 per cent acetic acid and stored in the same solution. To determine the pH gradient in the gel, the gel from the blank track (no protein applied in the well) of the same run was sliced into 0.5 cm pieces. Each slice was suspended in 1.0 ml double distilled water and crushed with a glass rod. The pH of the suspension was recorded. A plot was drawn of pH vs distance from the anode. The distance of the enzyme band was recorded and the pI of the nonglycoprotein AcPase was determined from the plot constructed.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Preliminary studies to determine optimum pH and optimum temperature of the crude enzyme extract obtained from the cotyledons of 3 days germination seedlings, indicated a pH optimum of 4.6 and temperature optimum of 60°C. However the enzyme was assayed at pH 4.6 and 37°C due to its instability at 60°C as confirmed by thermal stability studies of crude extract.

1. GERMINATION STUDIES:

Germination studies indicated that the specific activity of acid phosphatase (nkatals/mg protein) in cotyledons was maximum after 120 h of germination (Table 1).

The electrophoretic pattern obtained (pH 4.5 run) on staining for enzyme in the crude enzyme extracts revealed the presence of isozyme bands of AcPase of various intensities (Plate 1 and Figure 1). There were only two isoforms in the resting seed, while in the 24 h germinated seed, the same 2 isoforms slightly increased in their intensity with one additional,very minor form. From 48 h onwards till 120 h, 2 major and 2 minor isoforms were present, but the intensity of the major isoforms was maximum after 120 h of germination and the 2 bands were seen to have

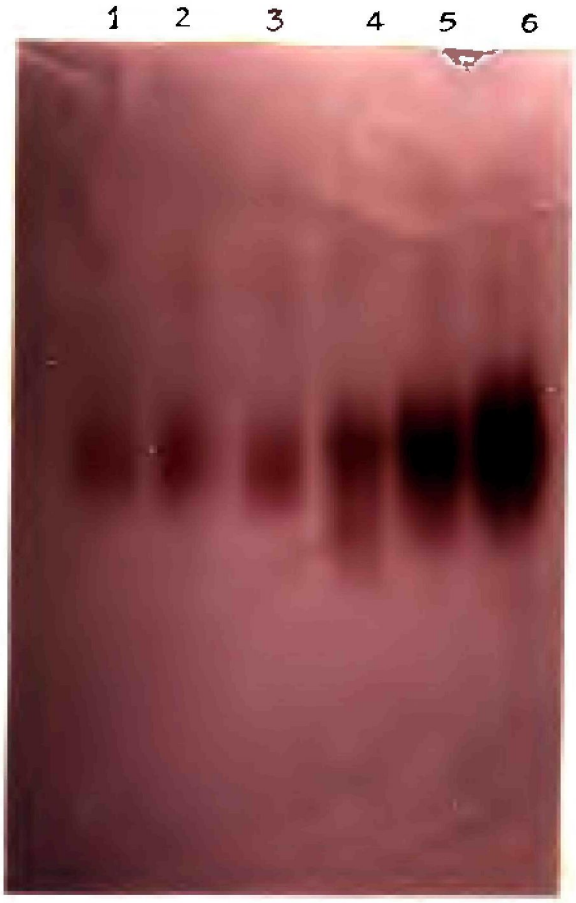
Table 1: AcPase activity in cotyledons of *Lablab purpureus* during germination

Particulars	Protein (mg/ml)	Enzyme Activity (n katal /ml)	Specific Activity (n katal /mg protein)
Resting seeds	20.5	6.0	0.30
24 h germinated seeds	22.0	10.0	0.45
48 h germinated seeds	15.0	15.0	1.00
72 h germinated seeds	16.0	25.3	1.58
96 h germinated seeds	16.0	33.3	2.08
120 h germinated seeds	16.5	55.3	3.40

PLATE 1 AND FIGURE 1 :

Lane 1: Resting seeds.
2: '24' h germinated seeds
3: '48' h germinated seeds
4: '72' h germinated seeds
5: '96' h germinated seeds
6: '120' h germinated seeds.

A nondenaturing disc PAGE at pH 4.5 (cathodic run) was performed and gel was stained for enzyme using Fast Red TR.



ENZYME STAIN

Plate 1 : Electrophoretic pattern of nonglycoprotein acid phosphatase at different stages of germination

- Light
- Medium
- Dark

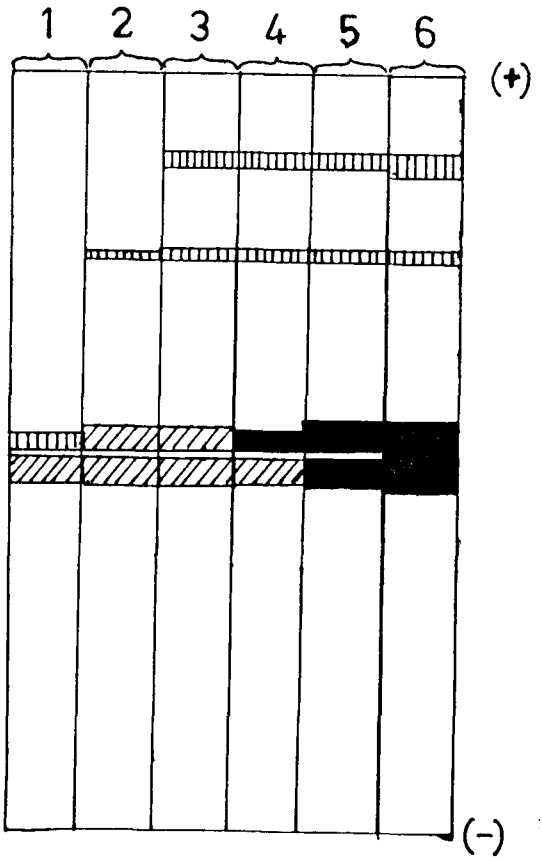


FIGURE 1

Electrophoregram of nonglycoprotein acid phosphatase at different stages of germination (enzyme stain)

merged into a thick band. Staining for glycoproteins revealed that none of the isoforms were glycoproteins.

ISOLATION AND PURIFICATION OF ACID PHOSPHATASE:

120 h germinated cotytledons were used as the material for the isolation of acid phosphatase since they showed maximum enzyme activity.

The results obtained by ammonium sulphate precipitation of crude enzyme extract followed by DEAE-Sephadex A-50 anion exchange chromatography, ultrafiltration using a 10 kDa filtermembrane and gel filtration on Sephadex G-100 and Sepharose-4B are presented in Table 2. The electrophoregram at all the steps of purification is shown in Plate 2 and Figure 2.

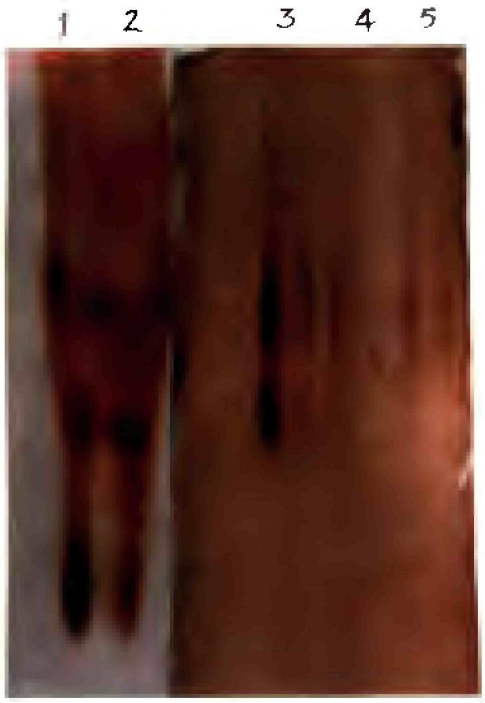
The elution profile of AcPase from the DEAE-Sephadex-A-50 column is shown in Figure 3. The unbound fractions did not show any enzyme activity, whereas the bound fractions having peak activity revealed four isozyme bands. Further, only the second enzyme activity peak in the elution profile was used for purification due to its high enzyme activity and low level of contaminating proteins as compared to the first peak.

Table 2: Purification of nonglycoprotein acid phosphatase from *Lablab purpureus* cotyledons

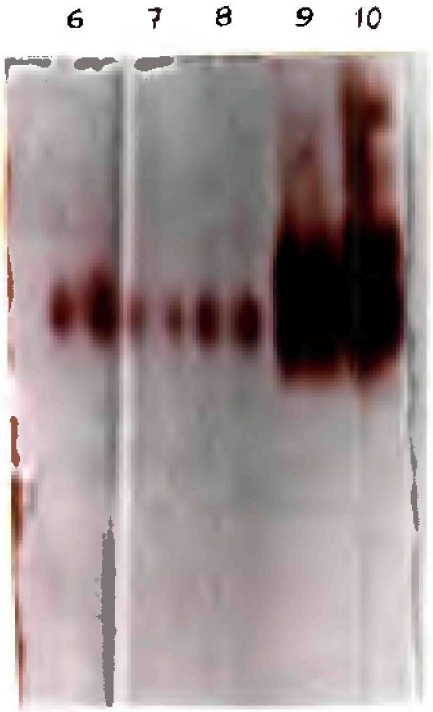
	Total volume (ml)	Protein (mg/ml)	Activity (n kat/ml)	Total activity (n kat)	Specific activity (n kat/mg protein)	Recovery (%)	Fold purification
Crude enzyme extract	110	12.0	48.3	5313	4.02	100	1.0
35-65% Ammonium sulphate precipitation	25.0	18.0	87.2	2180	4.84	41	1.2
DEAE-Sephadex-A-50 anion-exchange chromatography (bound peak fractions)	72.0	1.7	19.2	1382	11.29	26	2.81
Ultrafiltration	5.0	18.7	275.6	1378	14.74	25.9	3.67
Sephadex-G-100 (peak activity fractions)	15.0	2.13	86.5	1298	40.6	24.4	10.1
Sepharose 4B (peak activity fractions)	10.0	0.1	121.1	1210	1211.1	22.3	300.1

PLATE 2 AND FIGURE 2 :

Lane 1 and 10: 35-65 % ammonium sulphate fraction
2 and 9 : DEAE-Sephadex A-50 column pooled fraction (Second peak only)
3 and 8 : Ultrafiltered fraction
4 and 7 : Sephadex G-100 column pooled fraction
5 and 6 : Sepharose 4B column fraction (45-52)



SILVER STAIN



ENZYME STAIN

Plate 2 : Electrophoretic pattern of nonglycoprotein acid phosphatase at different stages of purification

- Very light
- Light
- Medium
- Dark

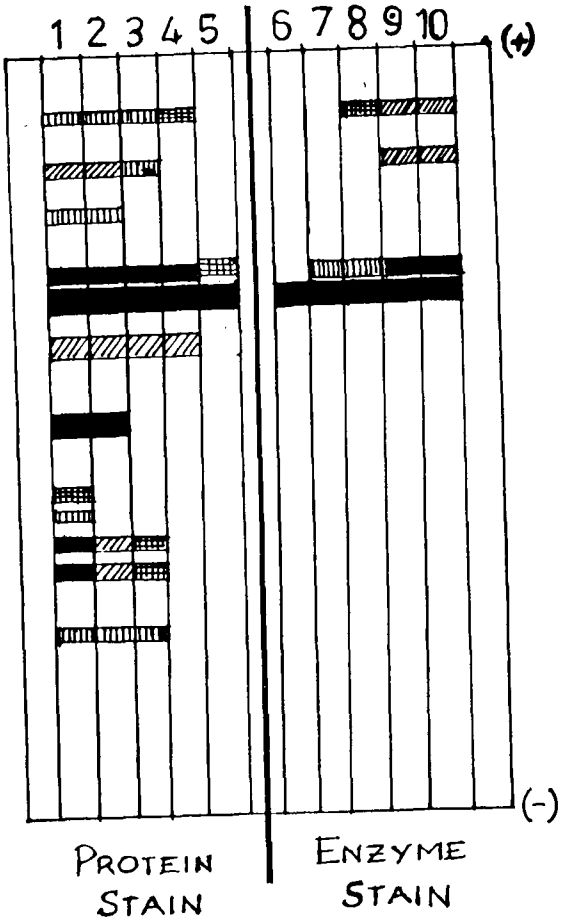


FIGURE 2

Electrophoregram of nonglycoprotein acid phosphatase at different stages of purification

Figure 3: Elution profile of field bean cotyledons nonglycoprotein acid phosphatase from DEAE-Sephadex A-50 column.

Purification of acid phosphatase from cotyledons of Lablab purpureus on DEAE-Sephadex A-50 column (2.0 * 25 cm) equilibrated with 100 mM Tris-HCl buffer, pH 7.2 and eluted the bound protein with a 0-0.3 N NaCl linear salt gradient using the same buffer. About 100 mg protein was applied on to the column and 2 ml fractions were collected.

Arrow mark indicates the start point of elution with the salt gradient.

●——● - Enzyme activity

○——○ -Protein (A_{280})

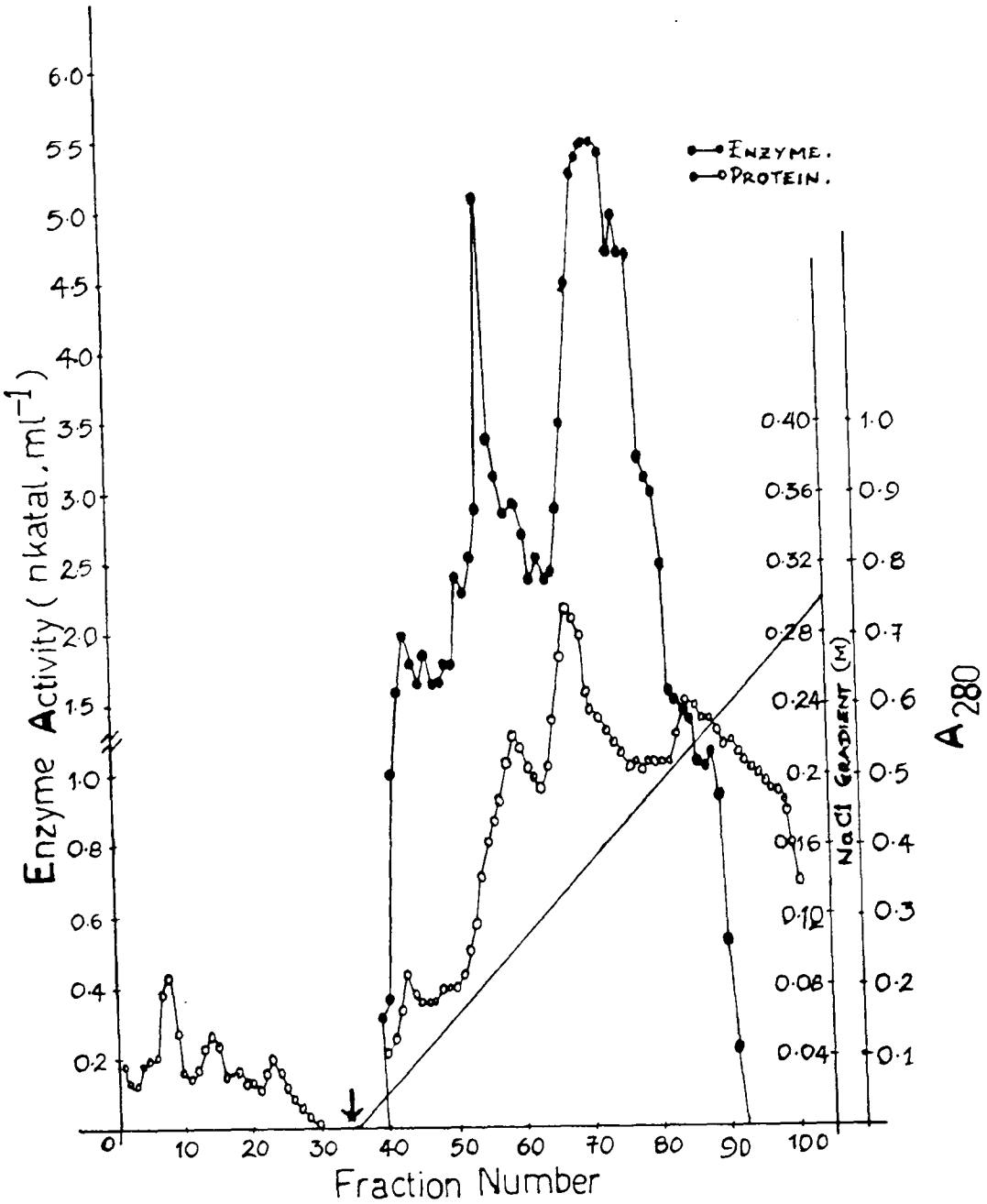

Enzyme
Protein.
Enzyme Activity (nkatal . ml^{-1})
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
80
90
100
Fraction Number
NaCl Gradient (M)
0.40
0.36
0.32
0.28
0.24
0.2
0.16
0.12
0.08
0.04
A_{280}
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

FIGURE 3

The peak activity fractions were concentrated using ultrafiltration and then applied on a Sephadex G-100 column and eluted as described earlier. The elution profile is as shown in Figure 4. The peak activity fractions concentrated using sucrose were applied on to a Sepharose-4B column and eluted as described earlier.The elution profile is as shown in Figure 5. A 300 fold concentration and a recovery of 22.3 per cent was obtained.

The peak activity fractions exhibiting a single enzyme band and a minor contaminating non-enzyme band (45-52) observed after performing a non-denaturing PAGE were pooled and used for further characterization of the enzyme.

HOMOGENEITY:

The partially purified enzyme preparation on electrophoresis at pH 4.5 revealed two protein bands on silver staining (Plate 2 and figure 2). The major protein band exhibited enzyme activity and minor contaminant protein did not exhibit any enzyme activity. Further purification was not attempted.

3. EFFECT OF pH:

From Figure 6, it is clear that the acid phosphatase showed a pH optimum of 4.8.

Figure 4: Elution profile of the nonglycoprotein acid phosphatase of field bean cotyledons from Sephadex G-100 column.

The peak activity fraction of peak II obtained from DEAE-Sephadex A-50 were pooled and ultrafiltered at 3 kg cm^{-2} pressure using a 10 kDa cutoff membrane. The concentrated fraction (45 mg protein) was loaded onto a Sephadex G-100 column (1.0 * 28 cm) equilibrated and eluted with 100 mM Tris-HCl buffer, pH 7.2. 3 ml fractions were collected and monitored at 280 nm and enzyme activity determined.

●——● -Enzyme activity

○——○ -Protein (A_{280})

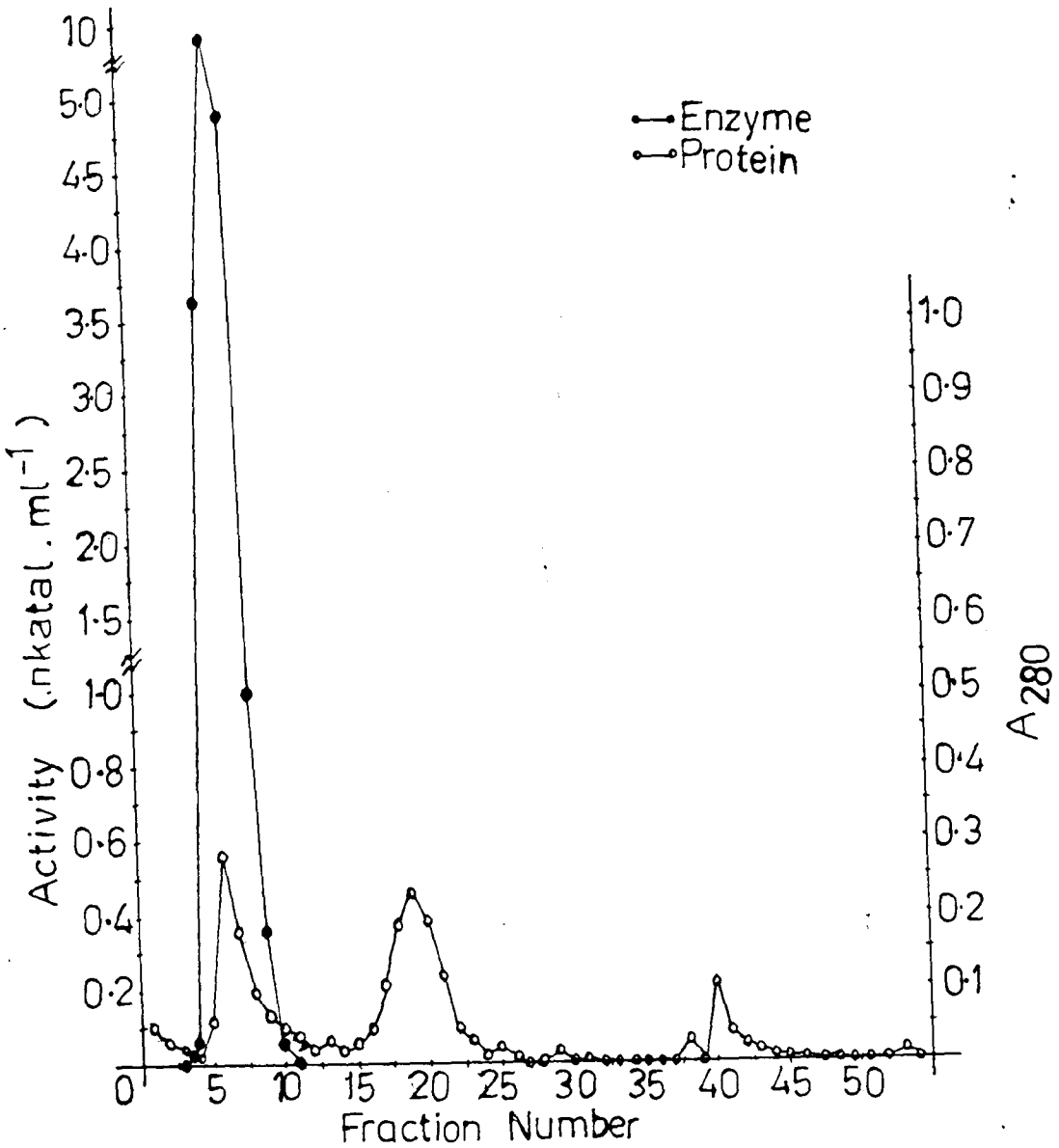

Enzyme
Protein
Activity (.nkatal.ml^{-1})
A_{280}
Fraction Number
10
5·0
4·5
4·0
3·5
3·0
2·5
2·0
1·5
1·0
0·8
0·6
0·4
0·2
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
5
10
15
20
25
30
35
40
45
50

FIGURE 4

Figure 5: Elution profile of the nonglycoprotein acid phosphatase of field bean cotyledons from Sepharose-4B column.

The peak activity fractions obtained from Sephadex G-100 column were pooled (20 mg protein) loaded onto a Sepharose-4B column (1.0 x 41 cm) equilibriated and eluted with 0.1 M Tris-HCl buffer, pH 7.2, 1.0 ml fractions were collected and monitored at 280 nm and enzyme activity determined.

—•—•— -Enzyme activity

—o—o— -Protein (A_{280})

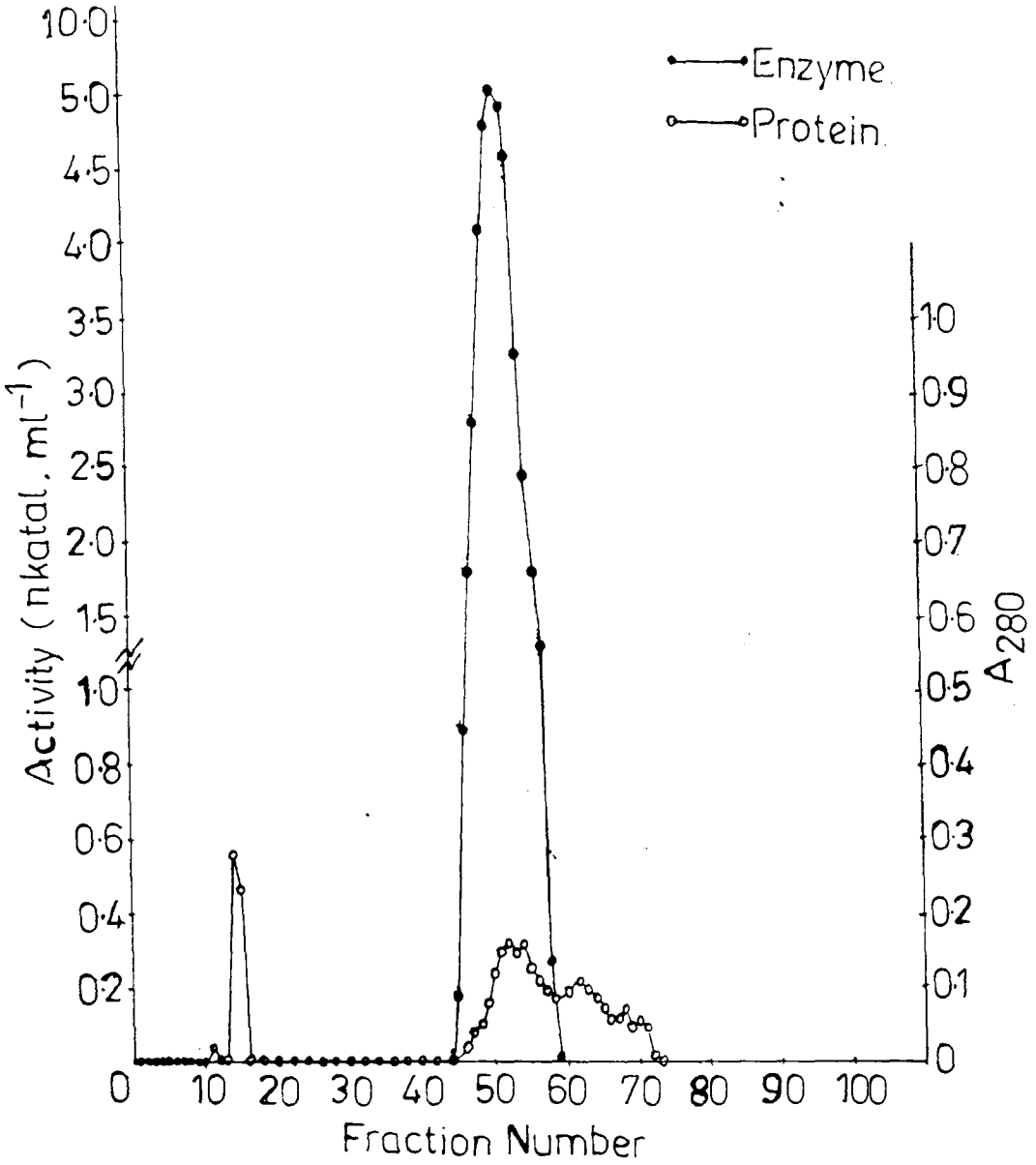

Enzyme
Protein
Activity (nkatal. ml^{-1})
A_{280}
Fraction Number
10·0
5·0
4·5
4·0
3·5
3·0
2·5
2·0
1·5
1·0
0·8
0·6
0·4
0·2
0
10
20
30
40
50
60
70
80
90
100
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0

FIGURE 5

Figure 6: pH optimum of field bean cotyledons nonglycoprotein acid phosphatase.

The enzyme was assayed at various pH values ranging from 3.0 to 6.2 using 200 mM citrate buffer, 6.4 to 7.0 - 200 mM Tris- malate and pH 7.2 to 8.0 - 200 mM Tris-HCl buffer. Acid phosphatase was assayed by the standard assay procedure. Enzyme activity was plotted against pH.

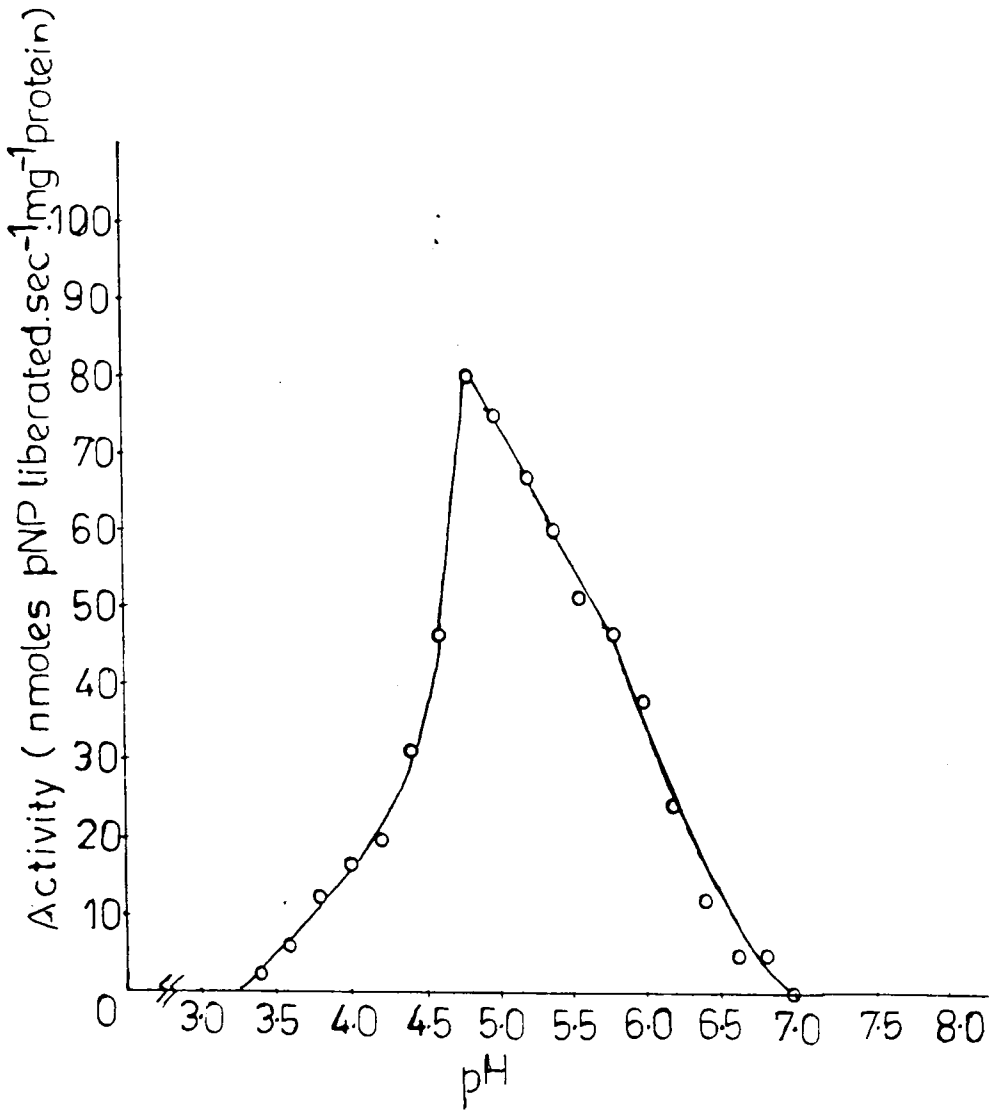

Activity (nmoles pNP liberated.sec-1mg-1protein)
100
90
80
70
60
50
40
30
20
10
0
3·0
3·5
4·0
4·5
5·0
5·5
6·0
6·5
7·0
7·5
8·0
pH

FIGURE 6

4. EFFECT OF TEMPERATURE AND THERMAL STABILITY:

The partially purified nonglycoprotein AcPase showed a temperature optimum of 55°C (Figure 7). The results of the studies on thermal stability of AcPase are as shown in Table 3. After 60 minutes of incubation at 37°C and at 45°C, the enzyme lost 89.5 and 71.7 per cent activity, respectively; whereas, at 55°C and 60°C, nearly 75 and 89.5 per cent of activity was lost after 20 minutes of incubation. There was no activity after 30 minutes of incubation at 60°C while no activity was observed after 10 minutes at 65°C.

5. EFFECT OF SUBSTRATE CONCENTRATION (K_m value):

The effect of substrate concentration on AcPase was studied using pNPP as substrate. The results are as shown in Figure 8A. The K_m value for pNPP was 4.24 mM as determined from the Lineweaver-Burk plot (Figure 8B). The V_{max} was 143 nmoles p-NP sec^{-1} mg^{-1} protein.

6. SUBSTRATE SPECIFICITY:

Different phosphate esters were used as substrates for field bean AcPase and their relative activities determined. These data are as shown in Table 4. The AcPase showed maximum activity with pyrophosphate disodium salt (250 per cent) and least activity with 5'-AMP (2.5 per

Figure 7: Temperature optimum of nonglycoprotein acid phosphatase from field bean cotyledons.

Enzyme activity was assayed using the standard assay procedure at different temperatures between 24°C and 70°C at pH 4.8.

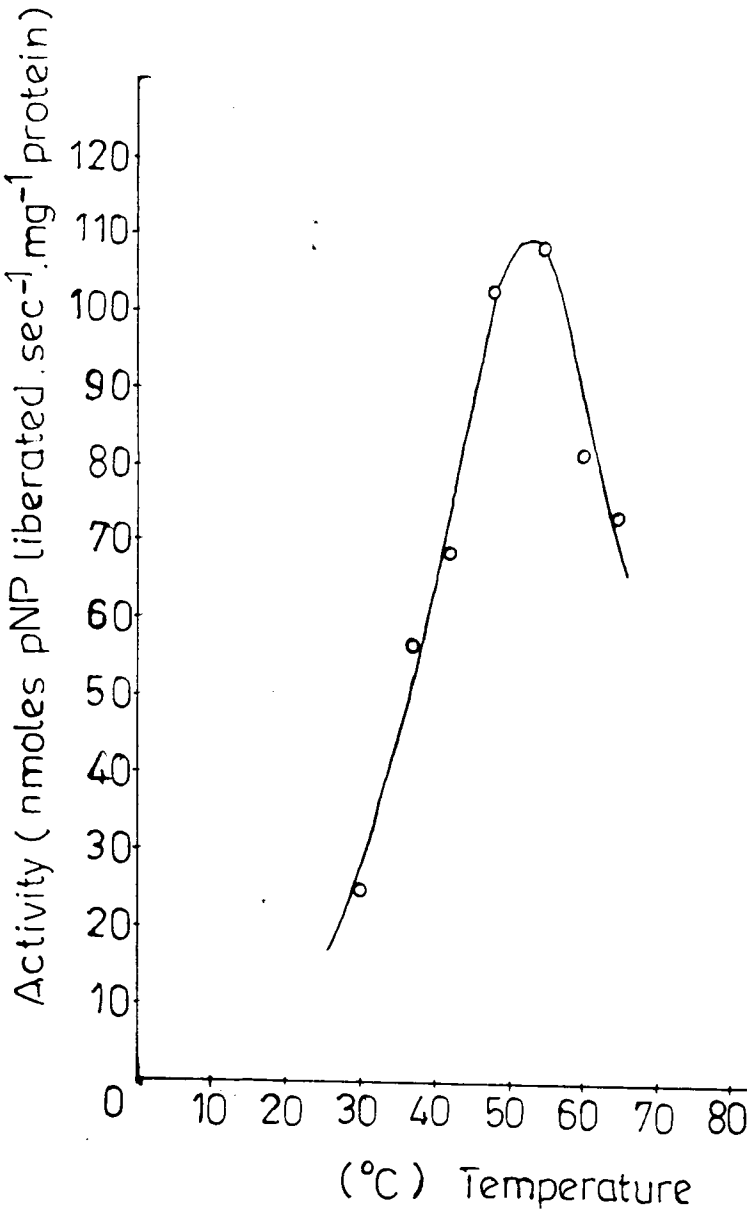

Activity (nmoles pNP liberated. sec-1. mg-1 protein)
120
110
100
90
80
70
60
50
40
30
20
10
0
10 20 30 40 50 60 70 80
(°C) Temperature

FIGURE 7

Table 3: Thermal stability of nonglycoprotein acid phosphatase from field bean cotyledons

Temperature (°C)	Residual activity of field bean cotyledons nonglycoprotein acid phosphatase at the following time intervals (minutes)					
	0	10	20	30	45	60
37	100.0	100.0	87.5	75.0	11.5	10.5
45	100.0	78.5	33.3	35.0	28.3	28.3
55	100.0	72.2	25.0	12.5	0.0	0.0
60	100.0	45.0	10.5	0.0	0.0	0.0
65	100.0	0.0	0.0	0.0	0.0	0.0

Figure 8A: Effect of substrate concentration on the rate of cotyledons nonglycoprotein acid phosphatase hydrolysis of pNPP.

1 mM to 35 mM pNPP was used. Activity (n moles pNP liberated per sec per mg protein) was plotted against pNPP concentration (mM).

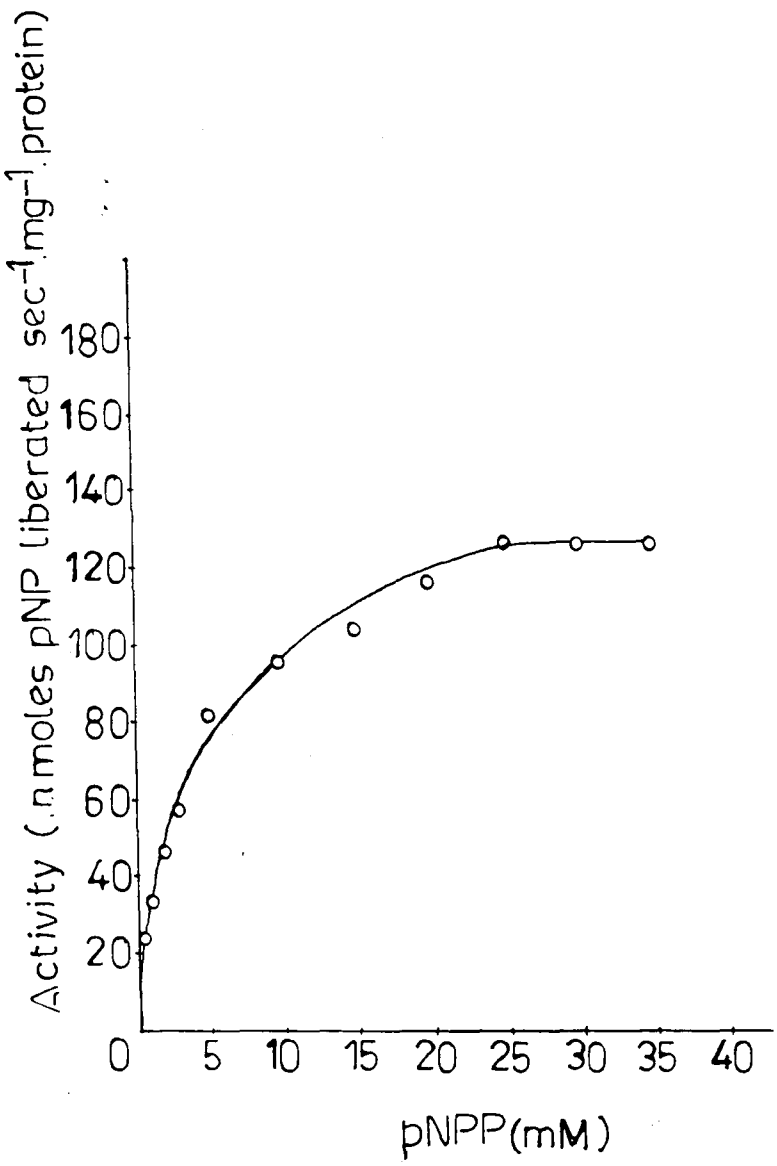

Activity (.n moles pNP liberated sec-1.mg-1.protein)
180
160
140
120
100
80
60
40
20
0
5
10
15
20
25
30
35
40
pNPP(mM)

FIGURE 8A

Figure 8B: Lineweaver-Burk plot for the estimation of K_m for field bean cotyleton nonglycoprotein acid phosphatase.

Data from substrate concentration curve was used for the double reciprocal plot.

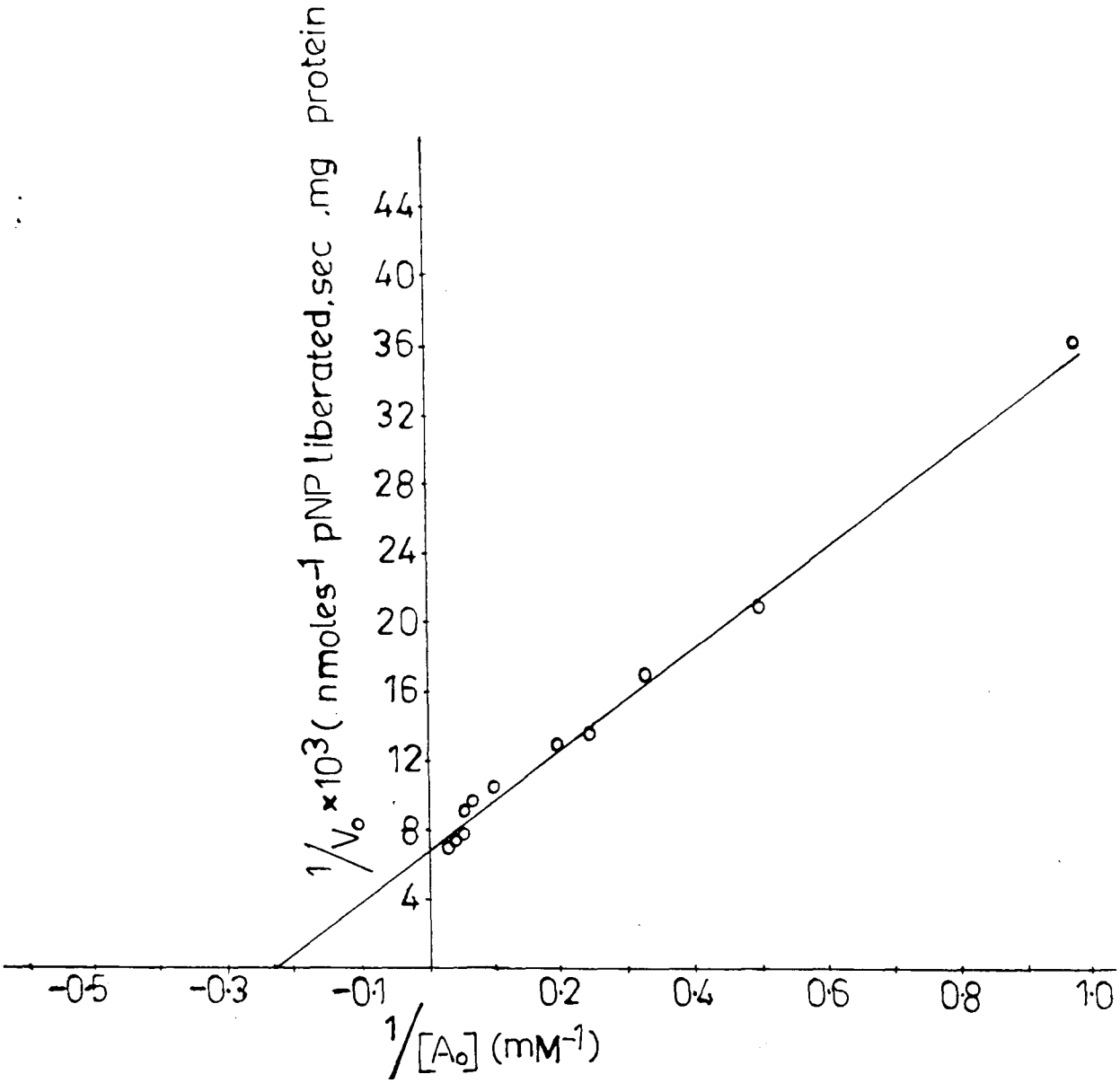

1/V₀ ×10³ (nmoles⁻¹ pNP liberated.sec .mg protein
44
40
36
32
28
24
20
16
12
8
4
-0·5
-0·3
-0·1
0·2
0·4
0·6
0·8
1·0
1/[A₀] (mM⁻¹)

FIGURE 8B

Table 4: Substrate specificity of field bean cotyledonary nonglycoprotein acid phosphatase

Sl. No.	Substrate	Relative activity (%)
1.	p-Nitrophenyl phosphate	100.0
2.	D-Glucose-1-phosphate	53.1
3.	D-Glucose-6-phosphate	19.5
4.	D-Fructose-1-phosphate barium salt	20.9
5.	D-Fructose-1,6-bisphosphate	4.6
6.	Sodium-ß-glycerophosphate	62.5
7.	Sodium phytate (from rice)	66.6
8.	Sodium pyrophosphate	250.0
9.	α-Naphthyl phosphate	7.2
10.	5'-AMP	2.5
11.	5'-ADP	10.3
12.	5'-ATP	125.0
13.	5'-UMP	7.5
14.	5'-CMP	7.2
15.	$NADP^+$	41.6
16.	O-Phospho-L-tyrosine	62.5
17.	O-Phospho-L-serine	52.2

cent). From the Table it can also be observed that the enzyme activity was low towards the nucleoside monophosphates and ADP, intermediate towards phosphoamino acids like O-phospho- L-serine and O-phospho-L-tyrosine and high towards ATP.

7. EFFECT OF METAL IONS AND VARIOUS REAGENTS:

The effect of various metal ions and reagents on AcPase activity is as shown in Table 5.

SDS was found to completely inactivate the enzyme. Inhibition was 78.8 per cent and 48.5 per cent with Hg^{2+} and sodium fluoride respectively. Among the metal ions, Hg^{2+} caused maximum inhibition. Inhibition by Ag^{+} and Co^{2+} was however, marginal (19.2 and 9.1 per cent, respectively). Sodium tungstate, Zn^{2+}, Cu^{2+} and Fe^{2+} were found to activate the enzyme to 118.2, 151.5, 151.5 and 127.3 per cent, respectively. Among the reagents KCN and PCMB were found to inhibit the enzyme to an extent of 39.4 and 33.6 per cent respectively, while sodium phosphate (dibasic) had no effect on the enzyme activity. Iodoacetic acid, 2-mercaptoethanol and D(+)-tartaric acid were found to increase the enzyme activity to 136,4, 170.0 and 181.9 per cent, respectively. The maximum increase was with Triton X-100 (197 per cent).

Table 5: Effect of metal ions and other reagents on the activity of field bean cotyledons nonglycoprotein acid phosphatase

Sl. No.	Metal ion/ reagent	Concentration	Relative activity (%)
1)	Control	--	100
2)	Silver nitrate	1mM	81.8
3)	Cobalt chloride	1mM	90.9
4)	Mercuric chloride	1mM	21.2
5)	Zinc chloride	1mM	151.5
6)	Copper sulphate	1mM	151.5
7)	Ferrous sulphate	1mM	127.3
8)	Magnesium chloride	1mM	167.0
9)	Sodium tungstate	1mM	118.2
10)	Sodium molybdate	1mM	100.0
11)	Sodium fluoride	1mM	51.5
12)	Sodium phosphate (dibasic)	25mM	100.0
13)	Potassium cyanide	1mM	60.6
14)	D (+) tartaric acid	1mM	181.9
15)	SDS	0.1%	0.0
16)	Triton-X-100	0.5%	197.0
17)	PCMB	1mM	66.4
18)	2-Mercaptoethanol	5mM	170.0
19)	Iodoacetic acid	1mM	136.4

8. DETERMINATION OF MOLECULAR WEIGHT:

Molecular weight determination on a Sepharose-4B column indicated that the molecular weight of AcPase was approximately 3,10,000 daltons as determined from the calibration curve constructed using standard proteins (Figure 9).

9. ISOELECTRIC FOCUSING:

Isoelectric focusing (IEF) of the partially purified AcPase revealed a single enzyme band on staining for the enzyme, with an isoelectric pH of 6.3 which was determined from the pH gradient plot (Figure 10A). The electrophoregram is shown in Plate 3 and Figure 10B.

Figure 9: Calibration curve for molecular weight determination by gel filtration of the nonglycoprotein acid phosphate from field bean cotyledons on Sepharose-4B.

Procedure and conditions are described in Chapter III. Plot of V_e/V_o against log molecular weight of standard proteins was drawn.

The standard proteins used were:

1) Carbonic anhydrase : M_r - 29,000
2) Bovine serum albumin : M_r - 66,000
3) Alcohol dehydrogenase : M_r - 1,50,000
4) Catalase : M_r - 2,32,000
5) Thyroglobulin : M_r - 6,70,000

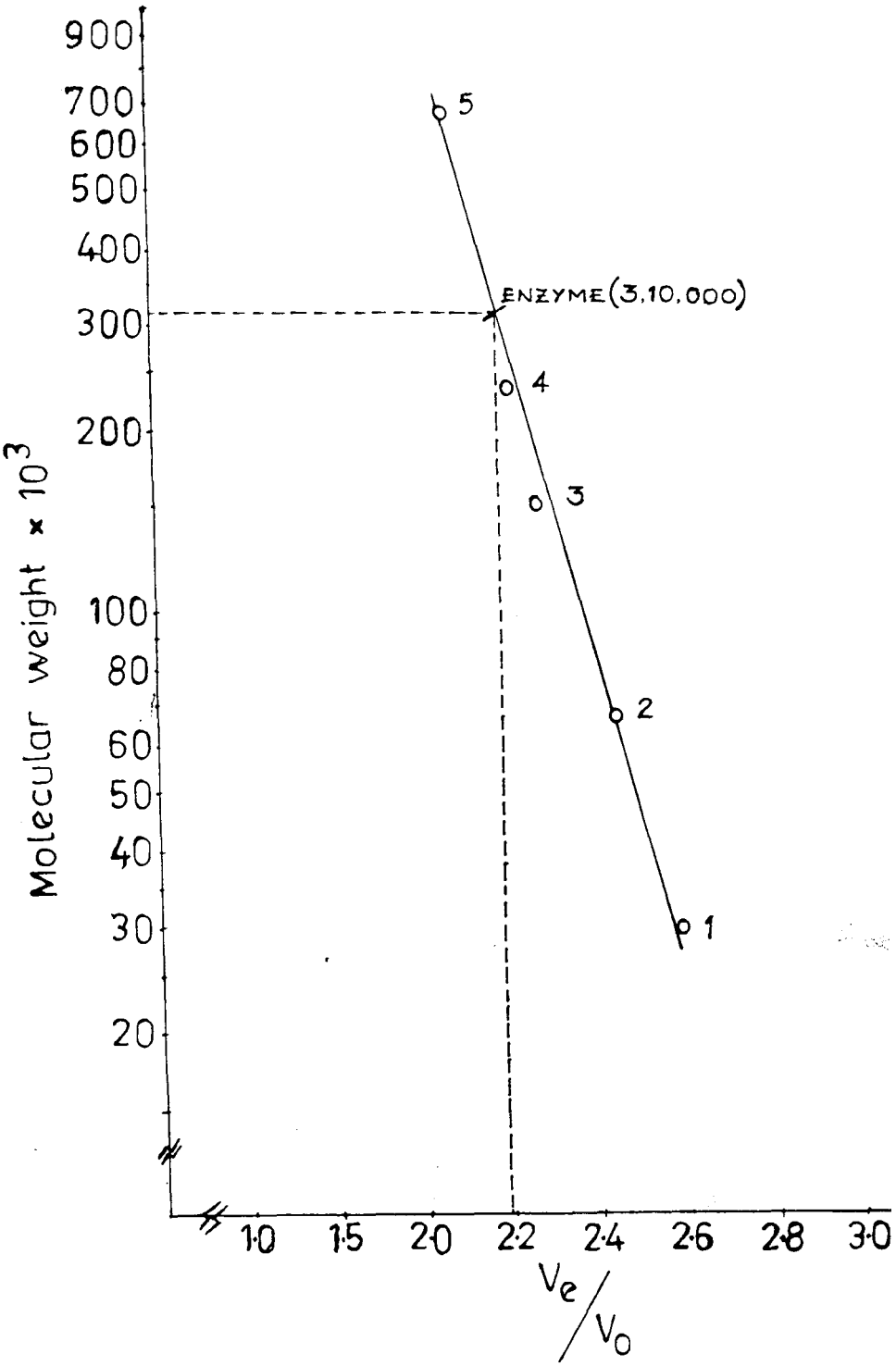

900
700
600
500
400
300
200
100
80
60
50
40
30
20
Molecular weight × 10^3
5
ENZYME (3,10,000)
4
3
2
1
1·0
1·5
2·0
2·2
2·4
2·6
2·8
3·0
Ve / Vo

FIGURE 9

Figure 10A: Determination of isoelectric pH of non-glycoprotein acid phosphatase from field bean cotyledons.

IEF was performed as described in Chapter III in 7.5 % gels containing ampholyte (pH range 3.0-10.0), pH gradient curve was established by plotting pH against the distance from the anode (mm).

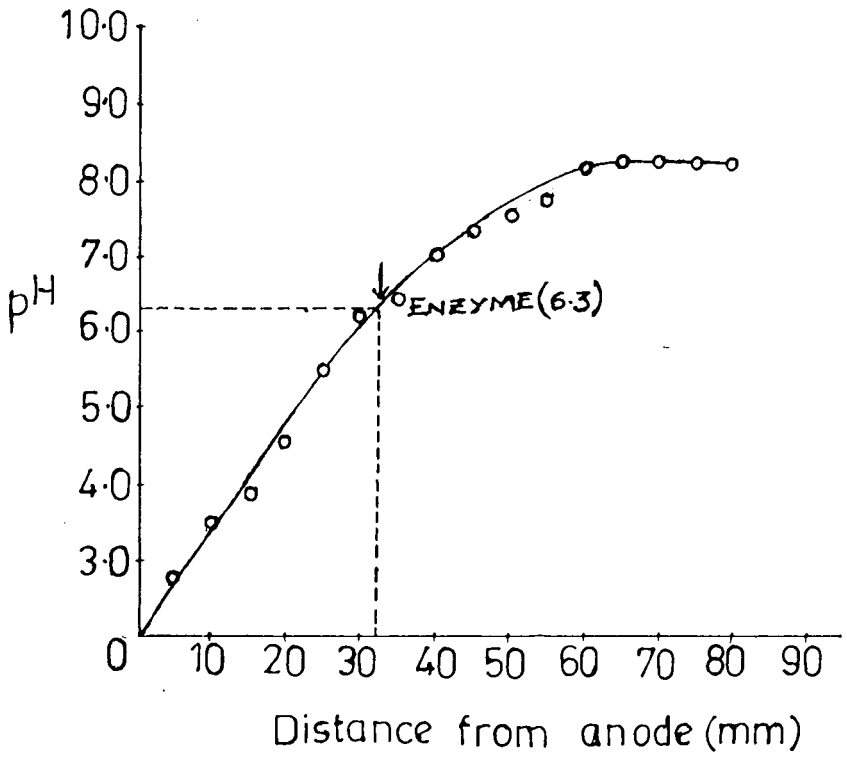

10·0
9·0
8·0
7·0
pH
6·0
5·0
4·0
3·0
0
10
20
30
40
50
60
70
80
90
ENZYME (6·3)
Distance from anode (mm)

FIGURE 10A

PLATE 3 :

IEF was performed as in Chapter III in 7.5 % gels containing ampholyte (pH 3.0-10.0). The gels were stained for enzyme as described in Chapter III.

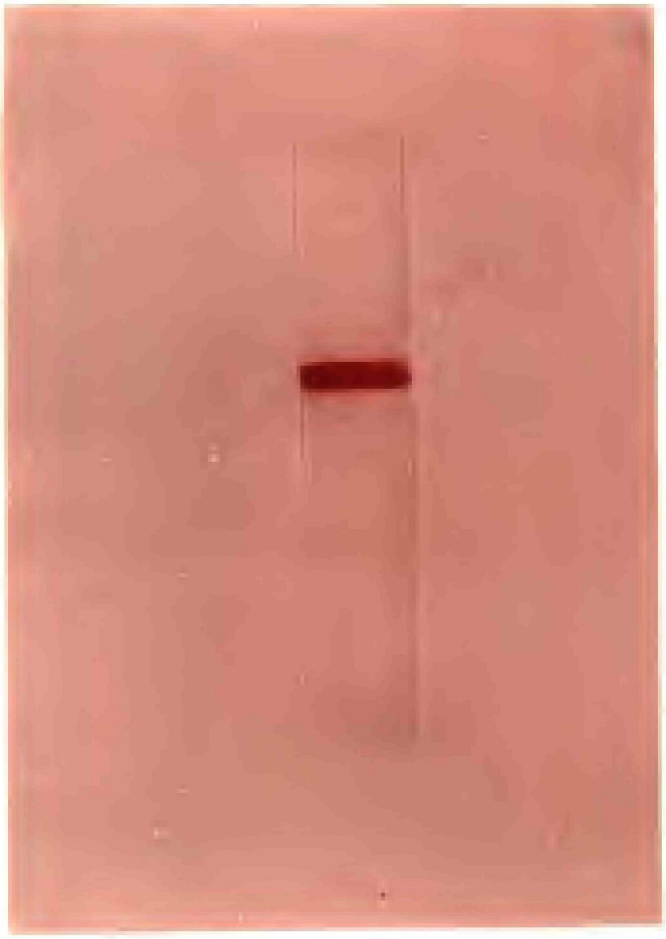


Plate 3 : Isoelectrofocussing pattern of nonglycoprotein acid phosphatase of field bean

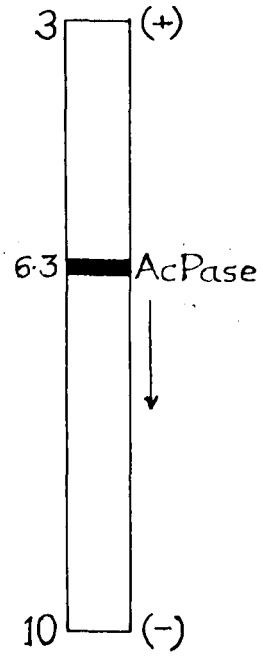


FIGURE 10B

Isoelectrofocussing pattern of nonglycoprotein acid phosphatase from field bean cotyledons obtained after gel filteration on Sepharose 4B column

DISCUSSION

V. DISCUSSION

Analyses of the data available on AcPases, indicate that the enzyme could be responsible for the mobilization of the phosphate during metabolism especially during cellular development and in proliferating embryonic cells.

The AcPase from cotyledons of 120 h germinated Lablab purpureus seeds was partially purified and characterized with the aim of assigning a possible physiological role to it.

1. GERMINATION STUDIES:

AcPase activity of Lablab purpureus cotyledons exhibited a gradual increase from 0 h to 120 h during germination. Similar results have been obtained in other seeds.

AcPase of wheat aleurone showed peak activity after 96 h germination (Clarke and Laidman, 1991) and an increase of activity of 4-6 fold was observed after 96 h of germination in Vigna sinensis seeds (Biswas and Cundiff, 1991). A 7-9 fold increase in the activity in the plumule and radicle of germinating winged bean seeds on fifth day of germination was observed (Kamala Devi and Madhusudanan, 1989).

This increased activity of AcPase could be to adequately supply phosphate to the rapidly dividing and growing cells of the embryo for the formation of nucleic acids, phospholipids, energy storage and generation. This could be one of the roles of AcPase.

2. PURIFICATION AND HOMOGENEITY:

The nonglycoprotein AcPase from the cotyledons of germinating field bean was purified using ammonium sulphate precipitation, ion-exchange chromatography, ultrafiltration and gel filtration. During, anion-exchange chromatography using DEAE-Sephadex A-50, the bound fractions showed four isoforms of the enzyme when eluted with a linear gradient of NaCl from 0-0.3 M. Ultrafiltration of peak DEAE-Sephadex A-50 fractions resulted in retention of 3 isoforms of AcPase. The concentrated fraction after ultrafiltration was subjected to Sephadex G-100 gel filtration and peak fractions from the Sephadex G-100 column were applied to a Sepharose-4B column. A single major isoform with a minor contaminating non-AcPase protein was revealed by non-denaturing electrophoresis at pH 4.5. The partially purified enzyme was not a glycoprotein as shown by the fact that PAS reagent did not stain the protein.

Similar purification procedures have been used for other AcPases. Four isoforms of AcPase were separated and purified from germinating seeds of Vigna sinensis using ammonium sulphate precipitation, DEAE-Cellulose column chromatography, Sephadex G-100 and G-200 gel filtration and phosphocellulose column chromatography (Biswas and Cundiff, 1991) while 2 isoforms were purified by alcohol precipitation and DEAE-Cellulose chromatography (De-Kundu and Banerjee, 1990). A major AcPase secreted by maize endosperm cultures was purified by ammonium sulphate precipitation and chromatography on columns of DEAE-Sephadex A-25, Con A-Sepharose and Sephacryl S-200 (Miernyk, 1992).

Glycoprotein AcPases have been purified using Con A-Sepharose from barley coleoptiles (Pasqualini et al., 1992), Japanese radish (Yashimoto et al., 1992), soybean root nodules (Sarath et al., 1993) and cotyledons of field bean (Vinayaka, 1992).

3. EFFECT OF pH:

The partially purified nonglycoprotein AcPase showed a pH optimum of 4.8 and is characteristic of AcPases which are active in the acidic pH range. Similar pH optimum was recorded by Yamagata et al. (1980) for the AcPase purified from the aleurone particles of rice grains. The AcPases of wheat germ (Parvin and Van Etten, 1991) and of plasma

membrane from peanut cotyledons showed a pH optimum of 4.7 (Basboa et al., 1987). A pH optimum of 5.0 was recorded in germinating Vigna sinensis seeds (Biswas and Cundiff, 1991), rice seedlings (Chen et al., 1992) and poppy seeds (Chung and Polya, 1992).

4. EFFECT OF TEMPERATURE AND THERMAL STABILITY:

The partially purified nonglycoprotein AcPase from cotyledons of 120 h germinated seeds exhibited a maximum activity at 55°C. It differs from the glycoprotein AcPase purified from 72 h germinated field bean cotyledon (Vinayaka, 1992) which has an optimum temperature of 48°C. The enzyme resembles other AcPases in optimum temperature. Of the multiple forms of AcPases isolated from germinating seeds of Vigna sinensis, the temperature optimum was 55°C for AP-I and IV while it was 60°C for AP-II (Biswas and Cundiff, 1991). The AcPase isolated from maize endosperm cultures exhibited a temperature optimum of 50°C (Miernyk, 1992) while the two isoforms of Vigna radiata seedlings showed an optimum of 60°C (De-Kundu and Banerjee, 1990).

The field bean cotyledonary nonglycoprotein AcPase was maximally stable at 37°C losing only 25 per cent activity after 30 minutes of incubation. At 45°C it lost 65 per cent activity under the same conditions and it was less stable at higher temperatures losing 75 per cent and 89.5 per cent of

its original activity at 55° and 60°C respectively after only 20 minutes. No activity was observed after 10 minutes of incubation at 65°C.

The glycoprotein enzyme from cotyledons of 72 h germinated seeds of field bean was also very stable at 37°C for a period of 60 minutes but lost 50 per cent of its activity between the temperature range of 45-60°C and 64 per cent activity at 65°C for the same incubation time (Vinayaka, 1992). The field bean axes nonglycoprotein AcPase was maximally stable at 30°C and 37°C, losing only 18 and 20 per cent of its activity after 60 minutes of incubation; but was less stable at higher temperatures losing 64 and 88 per cent of its activity at 45° and 60°C, respectively, for the same incubation time (Ramesh, 1994). The AcPase isoform AP- II from Vigna radiata lost 70 per cent of its activity at 55°C after 30 minutes of incubation (De-Kundu and Banerjee, 1990). The isoforms AP-II from tobacco lost 90 per cent of its original activity at 65°C after 30 minutes of incubation (Ninomiya et al., 1977).

The optimum temperature and thermal stability data on the partially purified field bean enzyme indicate that, though it has a high optimum temperature, it is not stable at that temperature as compared to the glycoprotein enzyme purified from cotyledons (Vinayaka, 1992) and nonglycoprotein enzyme from axes (Ramesh, 1994) from field bean seedlings.

5. EFFECT OF SUBSTRATE CONCENTRATION:

The field bean cotyledon nonglycoprotein AcPase exhibited Michaelis-Menten kinetics when assayed with varying pNPP concentrations. The enzyme showed a K_m of 4.24mM with pNPP, whereas the enzyme from axes (Ramesh, 1994) and glycoprotein enzyme from cotyledons of field bean (Vinayaka, 1992) showed K_m of 5 mM and 1.92 mM for pNPP, respectively. The K_m for pNPP varies from as low as 0.2μM for the peanut enzyme (Basha, 1984), to as high as 10 mM for the enzyme isolated from the plasma membrane of peanut cotyledons (Basboa et al., 1987). The tomato enzyme had a K_m of 2.9 mM (Tanaka et al., 1990) while the enzyme purified from wheat germ exhibited a K_m of 5.7 $\pm$ 0.95 and 1.9 $\pm$ 0.09 at pH 5.0 and 7.0 (Van Etten and Parvin, 1991).

The high K_m of 4.24 mM indicates that it does not have high affinity for pNPP. K_m values of AcPases purified from several other sources have very low K_m of 0.3 mM for pNPP as observed for AcPase from soybean suspension cultures (LeBansky et al., 1992) and AP-II of tobacco cells (Ninomiya et al., 1977). The K_m range for the four isoforms from *Vigna sinensis* was 0.11 mM-0.23 mM with pNPP as substrate (Biswas and Cundiff, 1991).

6. SUBSTRATE SPECIFICITY

The partially purified nonglycoprotein AcPase showed high activity with sodium pyrophosphate (250 %) and 5'-ATP (125 %) as substrates, but showed low activity with ADP, nucleoside monophosphates and sugar phosphates. Activity of 52.2 per cent and 62.5 per cent was observed when O-phospho-L-serine and O-phospho-L-tyrosine were used as substrates respectively. The cotyledonary glycoprotein enzyme, on the other hand, showed high activity towards phytate (608 %), ATP (478 %), glucose-1-phosphate (450 %) and pyrophospate (333 %) (Vinayaka, 1992). However, the purified nonglycoprotein AcPase had low affinity for phytate as substrate.

The two isoforms from Vigna radiata had no effect on phytate but showed high activity on pyrophosphate (De-Kundu and Banerjee, 1990). The sunflower AcPase recorded maximum activity with pyrophosphate followed by pNPP (Park and Van Etten, 1985), while the rye germ AcPase exhibited high activity towards ATP and pyrophosphate (Ferens and Morawiecka, 1985) with very low activity on phytate. Of the four isoforms of AcPase isolated from Vigna sinensis, AP-I and II showed maximum activity with pyrophosphate and AP-III and IV showed maximum activity with phosphoenolpyruvate. All four isoforms had no activity on phytate (Biswas and Cundiff, 1991). O-phospho-L-serine and O-phospho-L-tyrosine

were good substrates for potato tuber AcPase (Gellatly et al., 1993). The high activity of the AcPases from field bean towards pyrophosphate and ATP indicate that, they could be the two main sources of phosphate during germination of field beans.

7. EFFECT OF METAL IONS AND VARIOUS REAGENTS:

The partially purified cotyledonary AcPase was activated to different degrees by tungstate, Zn^{2+}, Cu^{2+}, Fe^{2+} and Mg^{2+} while it was inhibited by Ag^{+} (19 %), Co^{2+} (9 %) and Hg^{2+} (80 %) at 1 mM concentration. CN inhibited the enzyme activity by 40 per cent, while molybdate had no activity on the enzyme at 1 mM concentration.

Mg^{2+} and Mn^{2+} have been shown to activate AcPases from potato tuber (Gellatly et al., 1993), Japanese radish (Yashimoto et al., 1992) and rice seedlings (Chen et al., 1992). Ni^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} have been shown to activate the AcPase purified from Japanese radish (Yashimoto et al., 1992). Hg^{2+}, Cu^{2+} and molybdate inhibited strongly the AcPase from barley coleoptiles while Ca^{2+}, Ni^{2+}, Zn^{2+} and Rb^{+} activated the enzyme activity (Pasqualini et al., 1992). The enzyme from tomato leaves was activated by Zn^{2+}, Mg^{2+} and Mn^{2+} (Tanaka et al., 1990). Though molybdate was found to inhibit the enzyme activity of several sources, it had no effect on the partially purified enzyme from field beans.

The field bean nonglycoprotein cotyledonary AcPase was activated by PCMB, Triton-X-100, 2-mercaptoethanol, iodoacetic acid and D(+)-tartaric acid but was inhibited by F^-. The AcPase activity was unaffected by PCMB in peanut (Basha, 1984) and the two isoforms of AcPase from Vigna radiata were also not affected by PCMB (De-Kundu and Banerjee, 1990). This might indicate that free -SH groups are not required for supporting catalysis. However, the rye germ AcPase (Ferens and Morawiecka, 1985) and the four isoforms of AcPase from Vigna sinensis were inhibited by PCMB (Biswas and Cundiff, 1991) indicating the role of free -SH in catalysis. Triton-X-100, tartrate and Tween-80 were found to activate the enzyme from barley coleoptiles (Pasqualini et al., 1992). Tween-80, Triton-X-100 and sodium deoxycholate activated both the isoforms of Vigna radiata seedlings (De-Kundu and Banerjee, 1990) while F^- inactivated enzymes of potato tuber (Gellatly et al., 1993), rice seedlings (Chen et al., 1992), poppy seeds (Chung and Polya, 1992) and barley root AcPase (Panara et al., 1990b). ß-Mercaptoethanol and dithiothreitol activated the enzyme about 2.5-3 folds in embryoless half seeds of wheat (Saluja et al., 1989) of germinating chickpea seeds (Angosto et al., 1988) and of axes of field bean (Ramesh, 1994). ß-Mercaptoethanol had no effect on the enzyme from Vigna radiata (De-Kundu and Banerjee, 1990), peanut (Basha,

1984) and cotyledonary glycoprotein enzyme of field bean (Vinayaka, 1992). No conclusion can be drawn from the study regarding the active sites since PCMB and Hg^{2+} inhibit but, iodoacetic acid activates the enzyme.

The activation of the enzymes by detergents like Triton X-100 indicate that the enzyme could be cell bound and the release of which has further activated the enzyme. The activation of the enzymes by β-mercaptoethanol might possibly indicate that the cleavage of disulphide bridges could alter the native conformation to a more active conformation for the better hydrolysis of the substrate and thus the increase in activity. It could be also speculated that, it might be preventing oxidation of the -SH groups. The nonglycoprotein enzyme from field bean cotyledons was totally inactivated by SDS (0.1 %) which was similar to glycoprotein enzyme from 72 h germinated field bean cotyledons (Vinayaka, 1992). But SDS had no effect on the field bean axes AcPase (Ramesh, 1994). Sodium dodecyl sulphate also markedly affected the activities of the two isoforms AcPase from Vigna radiata (De-Kundu and Banerjee, 1990) and the four isoforms of AcPase isolated from Vigna sinensis were completely inhibited by 0.5 per cent SDS (Biswas and Cundiff, 1991).

8. MOLECULAR WEIGHT:

The molecular weight of the nonglycoprotein AcPase from cotyledons of 120 h germinated field bean seeds as determined by gel filtration on Sepharose-4B was 3,10,000. The glycoprotein AcPase from cotyledons was of M_r 1,25,000 (Vinayaka, 1992).

The peanut AcPase had a M_r of 2,40,000 and was found to be a homohexamer of subunit M_r 42,500 (Basha, 1984). The AcPase isolated from Shii-take had a M_r of 2,10,000 (Takako and Endo, 1987) while the AcPase from embryoless half seeds of wheat was of M_r 1,85,000 (Saluja et al., 1989).

9. ISOELECTRIC POINT (pI)

The isoelectric point of the nonglycoprotein AcPase partially purified from the field bean cotyledons was approximately 6.3. The glycoprotein AcPase from field bean had a pI of 5.4 (Vinayaka, 1992).

A pI of 6.28 was obtained for the major cytoplasmic isozyme purified from the barley root AcPase (Panara et al., 1990b). A pI of 6.7 for AcPase was recorded by chromatofocusing in cotyledons of germinating soybean seeds (Ullah and Gibson, 1988). The AcPases purified from plump and shriveled seeds of triticale had a pI of 5.9 (Ching et al., 1987).

The results obtained show that the nonglycoprotein AcPase from field bean cotyledons resembles other plant AcPases. There are also some similarities and some differences between this enzyme and the glycoprotein enzyme from field bean cotyledons and the nonglycoprotein AcPase from axes of field bean (Table 6). The most striking features of this enzyme is its high molecular weight which is higher than that reported for any other plant AcPase.

Table 6: Comparitive features of AcPases purified from cotyledons and axes of field bean

Source	24 h germinated cotyledons	72 h germinated cotyledons	120 h germinated cotyledons
Enzyme nature	glycoprotein	nonglycoprotein	nonglycorotein
pH optimum	5.2	5.4	4.8
Temperature optimum (^{o}C)	48	37	55
Thermal stability	stable at $37^{o}C$ for 60 min	stable at 30° & $37^{o}C$ for 60 min	Stable at $37^{o}C$ for 30 min
pI	5.4	-	6.3
Mol. wt. of native enzyme (daltons)	1,25,000	26,000	3,10,000
Activators	-	Triton-X-100	Triton-X-100, D(+)-tartaric acid, 2-mercapto-ethanol, iodo-acetic acid, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, tungstate
Inhibitors	Tungstate, Ag^{+}, Pb^{+}, Sodium phosphate (dibasic), SDS	Tungstate	SDS, PCMB, KCN, Ag^{+}, Co^{2+}, Hg^{2+}, F^{-}
K_m (mM) for pNPP	1.92	5.0	4.24

SUMMARY

VI. SUMMARY

The isolation, purification and characterization of a nonglycoprotein AcPase from the germinated cotyledons of Lablab purpureus was carried out with the view of assigning a physiological role to the enzyme. The results of the study are as summarized below and in table 7.

1. The AcPase was partially purified from 120 h germinated cotyledons of Lablab purpureus by ammonium sulphate precipitation, DEAE-Sephadex A-50 anion-exchange chromatography, ultrafiltration and gel filtration on Sephadex G-100 and Sepharose-4B. The fold purification was 300 and the recovery was approximately 22.3 per cent.

2. The enzyme preparation was partially pure with a minor contaminating protein band which had no enzyme activity. The major band exhibited AcPase activity and was not a glycoprotein.

3. The optimum pH and temperature were 4.8 and 55°C, respectively. The enzyme retained 75 per cent of its original activity after 30 minutes of incubation at 37°C but lost activity completely even after 10 minutes incubation at 60°C, indicating its low thermal stability.

4. The K_m value for the nonglycoprotein cotyledonary AcPase was 4.24 mM with pNPP as substrate.

5. The field bean nonglycoprotein AcPase displayed varying degrees of inhibition of when incubated with different metal ions and reagents, inhibition being total with 0.1 per cent SDS. Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, PCMB, 2-mercaptoethanol, D(+)-tartaric acid activated the enzyme. Triton-X-100 activated the enzyme maximally to 197 per cent. F^-, Hg^{2+}, Co^{2+}, and Ag^+ inhibited the enzyme.

6. The nonglycoprotein cotyledonary acid phosphatase showed low activity towards sugar phosphate and nucleoside mono- and di- phosphates. Sodium pyrophosphate (250 %) and ATP (125 %) were the preferred substrates. Activity was also observed with O-phospho-L-serine and O-phospho-L-tyrosine.

7. The molecular weight of the AcPase as determined by gel filtration on Sepharose-4B was approximately 3,10,000 kd.

8. The isoelectric point (pI) of the enzyme protein was approximately 6.3.

Table 7: Features of AcPase isolated from cotyledons of *Lablab purpureus*

1. Source	Field bean cotyledons (120 h germinated seeds)
2. No.of isoforms	4
3. AcPase purified	4th isoform (a nonglycoprotein)
4. Specific activity	1211 nkatal mg^{-1} protein
5. Fold purification	300
6. pH optimum	4.8
7. Temperature optimum	$55^{o}C$
8. Thermal stability	Stable at $37^{o}C$ for a duration of 30 minutes and unstable at higher temperatures
9. K_m (mM) for pNPP	4.24
10. Molecular weight of native enzyme	3,10,000 daltons
11. Isoelectric pH	6.3
12. Activators	Triton-X-100, D (+)-tartaric acid, 2-mercaptoethanol, iodo-acetic acid, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, tungstate
13. Inhibitors	SDS, PCMB, KCN, Ag^{+}, Co^{2+}, Hg^{2+}, F^{-}

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