BIOCHEMICAL STUDIES ON VACUOLAR ATPase OF LEGUMES: TONOPLAST ATPase FROM PEANUT

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By

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Gratis

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"BIOCHEMICAL STUDIES ON VACUOLAR ATPase OF LEGUMES : TONOPLAST ATPase FROM PEANUT"** in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biosciences and Biotechnology of the University is an authentic record of my own work carried out during a period from March, 1990 to September, 1995 under the supervision of **Dr. Vinay Sharma**, Reader, Department of Biosciences and Biotechnology, University of Roorkee.

The matter presented thesis has not been submitted by me for the award of any other degree of this or any other University.

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ABSTRACT

Tonoplast membranes possess a proton pumping ATPase. This enzyme generates proton gradient which acts as the driving force for transporting solutes across the membrane.

In this study peanut seedlings were used as the source of tonoplast membranes. ATPase associated tonoplast membrane fractions were isolated from seven-day-old seedlings by differential and 3-step sucrose-gradient centrifugation. The enzyme was solubilized by 5% Triton X-100. Phospholipid was essential for recovery of the tonoplast ATPase activity in presence of Triton X-100. The enzyme was purified through Sepharose CL-6B column by 27-fold over crude microsomal pellet. The purified enzyme was highly unstable compared to membrane associated enzyme. The Mr of ATP was estimated in the range from 400 kDa to 600 kDa. SDS-PAGE resulted in separation of three major polypeptides of 69, 55 and 20 kDa. Minor bands of 37 and 15 kDa were also co-purified.

Membrane bound ATPase was found to be sensitive to NO_3^- and exhibited a sharp pH optimum at 8.0. K_m and V_{max} values of the enzyme determined were 0.15 mM and 3.1 μmol Pi mg^{-1} protein h^{-1} respectively. The enzyme activity was inhibited by DTT, ß-mercaptoethanol and EDTA. The enzyme required divalent cations for its activity, Mg^{2+} being the most effective. The enzyme was stimulated by Cl^- ions. Monovalent cations, excepting RbCl, caused about two-fold stimulation in ATPase activity. Amongst various nucleoside phosphates, ATP was the most effective substrate. GTP was also partially hydrolysed.

Proton uptake by tonoplast vesicles was determined with acridine orange as pH probe. The proton pumping in tonoplast vesicles was inhibited (53%) by addition of 50 mM nitrate.

In *in vitro* experiment plant hormones like IAA, IBA, NAA, GA_3 and kinetin, in general,

showed inhibitory effect on the enzyme activity. In case of PAA, increase in enzyme activity was significantly higher than other hormones. In contrast, tonoplast ATPase showed either little or no inhibition in presence of ABA. In *in vivo*, all the hormones except GA_3 showed inhibitory effect.

In *in vitro*, effects of phenolic compounds on the enzyme activity were inhibitory excepting cinnamic acid and in presence of cinnamic acid marked increase in enzyme activity had been observed . Chlorogenic acid showed complete inhibition. In *in vivo* experiments seeds could not germinate in presence of phenolic compounds. However, stunted growth and with inappreciable enzyme activity could be observed in presence of hydroquinone at low concentrations.

Ultrastructure of tonoplast enzyme under electron microscope revealed the presence of head and stalk structures attached to the surface of the vesicles. Characteristic cleft was seen on the head piece of the enzyme structure. Head and stalk structures disappeared in presence of KNO_3. In contrast, when mitochondrial ATPase was compared to that of tonoplast, it was found that the enzyme lacked cleft and the enzyme structure remained associated with the membrane vesicles in presence of NO_3^-. Mitochondrial ATPase also differed from the tonoplast ATPase having shorter enzyme structure.

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(Soma Sen)

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ABBREVIATIONS

ABA - Abscisic acid

ADP - Adenosine 5'- diphosphate

AMP - Adenosine 5'- monophosphate

AO - Acridine orange

ATP - Adenosine 5'- triphosphate

CHAPS - (3-[(3-Cholamidopropyl) dimethyl ammonio]-1-propane sulphonate

CMC - Critical micelle concentration

CMP - Cytosine 5' - monophosphate

CTP - Cytosine 5' - triphosphate

d - day

DCCD - N,N'-dicyclohexylcarbodiimide

DIDS - 4-4'-diisothiocyano-2,2'-stilbene disulfonic acid

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

g - gram

GA_3 - Gibberellic acid

GDP - Guanosine 5'- diphosphate

GMP - Guanosine 5'- monophosphate

GTP - Guanosine 5'- triphosphate

h - hour

HEPES - N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

IAA - Indole-3-acetic acid

IBA - Indole-3-butyric acid

kDa - kilodalton

Kin - Kinetin

kV - kilovolt

M - molar

MES - 2-(N-morpholino)-ethanesulfonic acid

mg - miligram

mM - milimolar

min - minute

ml - mililitre

mm - milimeter

mV - milivolt

μg - microgram

μl - microlitre

μm - micrometer

μM - micromolar

μmol - micromole

NAA - Naphthylacetic acid

NBD-Cl - N' Chloro-4-nitrobenzo-2-oxa-1,3,-diazole

NEM - N-ethylmaleimide

nm - nanometer

PAA - Phenylacetic acid

p-NPP - *para*-nitrophenyl phosphate

PTA - Phosphotungstic acid

SDS - Sodium dodecyl sulphate

TEMED - N,N,N'N' - tetramethylethylenediamine

Tris - tris(hydroxymethyl)aminomethane

UDP - Uridine 5'- diphosphate

UTP - Uridine 5'- triphosphate

UMP - Uridine 5'- monophosphate

v/v - volume/volume

w/w - weight/weight

w/v - weight/volume

CHAPTER 1

1.0 INTRODUCTION

In higher plants, vacuoles occupy about 80-90% of the cell volume. They play an important role in the control of various cellular processes. These include the regulation of cell turgidity (Wagner, 1983), modulation of cytoplasmic component concentrations (Marty et al., 1980) and carrying out lytic function by virtue of their hydrolytic enzymes (Boller and Kenede, 1979). In some plant species they may also possess limited bio-synthetic capacity (Sharma and Strack, 1985). They act as storage organelles of acids, sugars, amino acids, alkaloids, phenolic compounds, vitamins and metabolites. Majority of these compounds are synthesized in cytosol. These are transported into the vacuole and are accumulated at concentration several times higher than cytosol. This process can occur only by the mediation of energy supplied by membrane bound ATPase (Higinbotham et al., 1974). It has now been established that tonoplast membranes possess a proton pumping ATPase that plays an important role in intracellular transport at the vacuole - cytosol interface by generating a proton gradient (Churchill et al., 1983; Lew and Spanswick, 1984; Uribe and Luttge, 1984; Sze, 1985). In addition, the enzyme may also maintain the vacuolar pH (Kurkdjian and Guerin, 1989). In a few plant species, the enzyme has been partially purified and some of its properties have been studied (Mandala and Taiz, 1985; Randall and Sze, 1986; Kaestner et al., 1988; Parry et al., 1989; Wang et al., 1989). However details on conformation of the enzyme and its active site residues have still not been worked out. Isolation and characterization of tonoplast associated H^+-ATPase would help in basic understanding of the mechanism of solute transport at tonoplast.

Peanut is an important crop of tropical climate and its seeds store about 31% proteins,

48% fats and 12% carbohydrates (Bewley and Black, 1983). However no work is available on tonoplast ATPase of peanut seedlings. So it was decided to isolate tonoplast membranes from developing peanut seedlings and characterize the bound ATPase. Thus for this Ph.D. thesis, following objectives were undertaken:

1) Isolation of tonoplast enriched membrane fractions and associated ATPase enzyme.
2) Purification and biochemical characterization of the enzyme.
3) Electron microscopic study of the enzyme.
4) Study of effects of plant hormones and phenolic compounds on tonoplast ATPase.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Tonoplast

2.1.1. General considerations

Mature cells of a higher plant contain two principal compartments. One is outer cytoplasmic compartment which is surrounded by plasmalemma and the other is inner compartment *i.e.* vacuole which is encased by another membrane, the tonoplast. Mature vacuoles are multi-functional organelles. Higher plant vacuoles were first isolated and biochemically characterized by Matile (1975, 1978). Earlier work on plant vacuoles was mainly concerned with the role of vacuole in cell turgidity (Wagner, 1983; Boller and Wiemken, 1986), maintenance of ion balance, storage and sequestration of ion and metabolites (Marty et al., 1980), lytic functions (Boller and Kenede, 1979; Boudet et al., 1981) etc. Recent researches focussing on the transport mechanism of tonoplast have generated new avenues to know more about the functions of the mature vacuoles of higher plants. Previously no marker enzyme for the tonoplast was known, hence to characterize the vacuolar membrane, it had to be obtained from separated and pure vacuoles. Vacuoles had been prepared from lysed protoplasts of various tissues using i) osmotic lysis (Wagner and Siegelman, 1975), ii) polybase-induced lysis (Buser and Matile, 1977) and iii) lysis due to shear under isotonic conditions (Lorz et al., 1976). The main problem in preparing vacuoles by lysis of protoplasts is the association of vacuoloplasts with the tonoplast. Vacuoloplasts are the vacuoles which are surrounded by the plasmalemma in addition to the tonoplast and contain few cytoplasmic remnants between the membranes (Lorz et al., 1976). Pure vacuoles not containing vacuoloplasts were first obtained directly from the red beet

by slicing root tissue into plasmolysing medium (Leigh and Branton, 1976; Admon et al., 1981) and from the latex of *Hevea brasiliensis* (d'Auzac et al., 1982). Later vacuolar membranes, not containing vacuoloplast have been obtained by many workers (Bennett et al., 1984; Poole et al., 1984; Mandala and Taiz, 1985; Pugin et al., 1986) from microsomal fractions separated on density gradient. These workers have shown that the characteristics of the separated membrane fractions are similar to those of tonoplast obtained from vacuoles and thus a tonoplast enriched light microsomal fraction from microsomes has been identified.

2.1.2 Isolation

To isolate tonoplast it is necessary initially to separate the mitochondria from microsomes by differential centrifugation. Centrifugal forces of 10,000x g for 20 min and 13,000x g for 15 min have pelleted 90% of the mitochondrial marker (Hodges and Leonard, 1974; Leonard and Vanderwoude, 1976; DuPont et al., 1982; Nagahashi and Hiraike, 1982; Giannini and Briskin, 1987). Microsomal pellet has been obtained by centrifugation of 13,000x g supernatant at the speed of 80,000x g for 30 min (DuPont et al., 1982; Poole et al., 1984). Principal method for separating tonoplast fractions in microsomal pellet from other membrane fractions like plasmalemma, endoplasmic reticulum and Golgi apparatus is isopycnic centrifugation. The density gradient used in this centrifugation covers the density range which necessarily includes the density of the macromolecules. After proper time the macromolecules are concentrated at position in the gradient where their density is equal to the density of the sedimentation medium. Sucrose step density gradient has been used by many workers to isolate tonoplast fractions. Struve and Luttge (1987) have layered microsomal suspension on three step sucrose gradient - 24% / 37% / 50% and centrifuged at 80,000x g for 2 h. Lew and Spanswick (1984) have used 20, 34 and 42% sucrose (w/w) where tonoplast has banded at 20 / 34% interface. DuPont

(1987), Jochem and Luttge (1987) and Chanson and Pilet (1989) have used 10% / 35% /45%, 22% / 30% / 40% and 20% / 30% / 60% sucrose gradient respectively to isolate tonoplast from microsomal fraction. Dorp et al. (1986) have used 0.48, 0.78 and 1.2 M sucrose for discontinuous gradient. Many workers like Poole et al. (1984), Blumwald and Poole (1985), Kasamo (1986) and Giannini and Briskin (1987) have used two step sucrose density gradient where tonoplast is isolated at 10% / 23-26% interface. Continuous sucrose gradient (15 to 45 / 60% sucrose) has been employed by Lin et al.(1977), Briskin and Leonard (1980) and DuPont et al. (1988). 25% sucrose cushion has been used by Mariaux et al. (1994) and Ratajczak (1994).

Dextran rather than sucrose has also been used as a linear density gradient to isolate tonoplast. In mung bean seedlings, Wang et al. (1989) have collected tonoplast at 0 / 4% dextran interface. Dextran density gradient has also been used in oat roots (Churchill et al., 1983; Churchill and Sze, 1983; Kaestner and Sze, 1987). In pear fruit (Hosaka et al., 1994) 6% dextran cushion has been used for isolation of tonoplast.

2.1.3 Density

Tonoplasts band at a density of 1.10 - 1.13 g/cm^3 (Boller and Kenede, 1979; Briskin and Leonard, 1980; DuPont et al., 1982; Poole et al., 1984; Scherer and Fischer, 1985; DuPont, 1987; Oleski et al., 1987a; Struve and Luttge, 1987).

2.1.4 Tonoplast bound enzymes

Two enzymes have clearly been identified as bound to the tonoplast: a H^+-ATPase (Walker and Leigh, 1981a; Sze, 1985) and a pyrophosphatase (Karlsson, 1975; Walker and Leigh, 1981b; Wagner and Mulready, 1983; Rea and Poole, 1984).

2.2 Tonoplast H^+-ATPase

2.2.1 Isolation

As tonoplast ATPase is bound to the membrane, the enzyme can be isolated by the method, applied for the isolation of the tonoplast. Various methods of isolation of tonoplast are discussed in section **2.1.2**. The band of nitrate sensitive ATPase is found associated with tonoplast enriched fraction obtained at sucrose concentration of 25 to 28% or less (around 1.10 g/cm^3) (Poole et al., 1984). Sen and Sharma (1994) have used 3-layers of 15, 35 and 45% sucrose (w/v) for separating tonoplast fractions from microsomal pellet and 15 / 35% sucrose interface has been found to be enriched in tonoplast bound ATPase.

2.2.2 Solubilization

Triton X-100 has been used as most useful detergent to solubilize tonoplast ATPase (Lai et al., 1988; Parry et al., 1989; Hosaka et al., 1994). Bowman et al. (1987) have solubilized the enzyme in two detergents - Triton X-100 and N-hexadecyl-N,N-dimethyl-3-amino-1-propane sulfonate. Mandala and Taiz (1985) and Wang et al. (1989) have solubilized the enzyme in two step procedure - first 0.15% deoxycholate to remove 60% of the protein, and then 40 mM octylglucopyranoside has been used to solubilize the ATPase. Ratajczak (1994) has used Brij 58 as the detergent for solubilizing the tonoplast ATPase.

2.2.3 Purification

Solubilized enzyme has been purified on linear sucrose gradient by Mandala and Taiz (1985, 1986), Bowman et al. (1987), Wang et al. (1989). Randall and Sze (1986) have purified the enzyme by gel filtration chromatography using Sepharose 4B column. Lai et al. (1988) have used Sepharose CL-6B column for chromatography. Parry et al. (1989) have used Sephacryl S-400 and anion exchange FPLC (first protein liquid chromatography) on Mono-Q for purification

of the tonoplast ATPase.

2.2.4 General properties

It has already been established that tonoplast H^+-ATPase has a pH optimum around 8.0 (Aoki and Nishida, 1984; Smith et al., 1984a). However, Kawata and Yoshida (1988) have reported a lower pH optimum of 7.0. Leigh and Walker (1980a) and Poole et al. (1984) have described pH optimum between 7.5 and 8.5. Jochem and Luttge (1987) have found a pH range between 6.5 to 8.0 for the optimum activity of H^+-ATPase.

K_m value varies from species to species. Usually it is below 0.5 mM in beet root (Bennett et al., 1984), oat root (Randall and Sze, 1986) and *Kalanchoe* (Smith et al., 1984a). However, Kasamo (1986) and Kawata and Yoshida (1988) have reported higher K_m value (0.6 - 0.8 mM) in *Phaseolus mungo* and *Vigna radiata* respectively.

2.2.5 Molecular weight and subunits

Tonoplast H^+-ATPase is a multimeric enzyme consisting of combination of peripheral and integral membrane subunits. Functional size of the holoenzyme determined by gel filtration is 300 - 500 kDa (Randall and Sze, 1986). Radioactive analysis has also indicated that the functional molecular weight of the tonoplast ATPase is around 400 kDa (Mandala and Taiz, 1985; Wang et al., 1989). SDS-PAGE has led to identification of two major polypeptides 66-74 kDa and 54-64 kDa in yeast (Kane et al., 1989), *Neurospora* (Bowman et al., 1987), oat (Lai et al., 1988), corn (Mandala and Taiz, 1985), maize (Chanson and Pilet, 1989), mung bean (Wang et al., 1989). Mandala and Taiz (1986) have characterized the subunit structure of the tonoplast ATPase with help of both immunological and inhibitor binding site. Antibodies to the 72 kDa polypeptide and the native enzyme both strongly inhibit enzyme activity. [^{14}C]NBD-Cl preferentially has labeled the 72 kDa, and the labeling is prevented by ATP. The result indicates

that the 72 kDa is the catalytic domain of enzyme. [^{14}C] DCCD is a inhibitor of the proton channel portion of mitochondrial ATPase. It binds to the 16 kDa subunit, which may comprise the proton channel. 62 kDa is the binding site of nucleotide which suggests that it may function as regulatory subunit. Randall and Sze (1986) have found in oat root that 72 kDa and 60 kDa are copurified with enzyme activity and 14-18 kDa subunit is DCCD binding peptide. Minor component of 40 kDa and 13 kDa are also copurified with ATPase activity. Bowman et al. (1989) have found in *Neurospora* that 16 kDa peptide is integral part and 72 and 62 kDa peptides are peripheral part of the enzyme which could be dissociated by the treatment of NO_3^-. According to Forgac (1989) two nucleotide-binding subunits of 69 and 60 kDa, associated with peripheral V_1 complex and 16 kDa subunit, associated with integral V_0 complex, are the three major subunits common to all V-type ATPases. Parry et al. (1989) have described that tonoplast H^+-ATPase constitutes 6-7 major polypeptides - 67, 55, 52, 44 and 32 kDa are peripheral sectors and 100 and 16 kDa polypeptides are integral sectors. According to DuPont and Morissey (1992) and Ward and Sze (1992), in plants, as many as 10 different polypeptides contribute to the subunit composition of the tonoplast H^+-ATPase.

2.2.6 Effectors and Inhibitors

2.2.6.1 Divalent cation requirement

Ionic species like ATP^{4-}, $HATP^{3-}$ ($HATP^{3-} \rightarrow ATP^{4-} + H^+$) form complexes with Mg^{2+}. This bound form of ATP with Mg is the true substrate for ATP hydrolysis. Mg^{2+} dependency of tonoplast H^+-ATPase has been described by many workers (Lin et al., 1977; Doll et al., 1979; Leigh and Walker, 1980a; Aoki and Nishida, 1984; Poole et al., 1984; Thom and Komor, 1984a; Smith et al., 1984a; Struve and Luttge, 1987). The activity of the ATPase is highly specific for Mg ATP^{2-}; the other ionic states of ATP like ATP^{4-}, $HATP^{3-}$, $MgHATP^-$ and

Mg_2ATP are not stimulated (Thom and Komor, 1984b). Order of effectiveness of divalent cations is $Mg^{2+} \geq Mn^{2+} > Ca^{2+} \geq Zn^{2+}$ (Jochem et al., 1984; Kasamo, 1986; Kawata and Yoshida, 1988; Wang et al., 1989). O'Neill et al. (1983) have reported that the tonoplast H^+-ATPase is stimulated most effectively by Mn^{2+}. Walker and Leigh (1981a) and Sen and Sharma (1994) have found that in the absence of KCl the order of effectiveness of divalent cations changes with Mn^{2+} being the optimal.

2.2.6.2 Monovalent cation requirement

Monovalent cations have no significant effect on tonoplast H^+-ATPase. The order of effectiveness is $NH_4^+ > Na^+ > K^+$. This cation effect is abolished when the membrane is treated with 0.01% (v/v) Triton X-100 (Walker and Leigh, 1981a; Jochem et al., 1984).

2.2.6.3 Anion requirement

Tonoplast H^+-ATPase is stimulated by Cl^- (Poole et al., 1984; Smith et al., 1984b; Mandala and Taiz, 1985; Oleski et al., 1987a; Struve and Luttge, 1987). The order of effectiveness of monovalent anions is KCl $\geq$ KBr $\geq$ KI (Kasamo, 1986).

2.2.6.4 Inhibitors

NO_3^-, DCCD, NBD-Cl, NEM, DIDS are the most effective inhibitors of tonoplast ATPase (Randall and Sze, 1986). NBD-Cl reacts with either cysteine or tyrosine residues on the catalytic subunit of the ATPase. NEM interacts with a sulfhydryl group in the catalytic subunit that constitutes an ATP-binding site (Randall and Sze, 1986). DCCD inhibits both ATPase activity and H^+ pumping. The site of DCCD inhibition on the ATPase resides within a hydrophobic environment (Randall and Sze, 1986). In the absence of Cl^-, DIDS inhibits the formation of pH gradient and also inhibits ATPase activity (Churchill and Sze 1984). According to Bowman et al. (1989) and Dschida and Bowman (1995), nitrate inhibits the enzyme activity

by dissociating peripheral polypeptides from the integral membrane part of the enzyme. They have observed under the electron microscope that the treatment with nitrate removes the head and stalk structure from the vacuolar membrane. Usually plasma membrane associated H^+-ATPase is sensitive to vanadate. Wagner and Mulready (1983), Henry and Pilet (1988) and Montrichard et al. (1989) have found vanadate sensitive ATPase associated with vacuoles of *Tulipa* petals, *Rubus* cells and in *Acer pseudoplatanus* respectively. According to Cocucci (1986) erythrosin B and rose bengal inhibit 85% ATPase activity of both plasmalemma and tonoplast. Moriyama and Nelson (1988) have found that fusidic acid and suramin inhibit tonoplast ATPase activity. Shu-I-Tu et al. (1988) have reported that Hg^+ in μM concentration inhibits tonoplast ATPase activity. Some herbicides like oryzalyn and oxyfluorfen inhibit tonoplast ATPase (Ratterman and Balke, 1987, 1988). Colombo et al. (1991) have reported that the fungicides like penconazole and flusilazol affect tonoplast H^+-ATPase. Giannini et al. (1990 and 1991) have described that the glyceollin, a phytoalexin of *Glycine max* inhibits 90% of the tonoplast ATPase activity.

2.2.7 Substrate specificity

ATP is the most effective substrate of the tonoplast ATPase. GTP is also partially purified (Walker and Leigh, 1981a; Smith et al., 1984a; Mandala and Taiz, 1985; Kasamo, 1986; Kawata and Yoshida, 1988). Poole et al. (1984) and Thom and Komor (1984a) have reported more than 70% hydrolysis of GTP and UTP by tonoplast ATPase. Wang et al. (1989) have found that GTP and UTP are the most effective substrates for membrane bound ATPase but the soluble ATPase is hydrolysed 23% and 31% by GTP and UTP respectively.

2.2.8 Thermal stability

According to Poole et al. (1984) tonoplast membrane frozen in liquid N_2 can be stored

at -70°C. Freezing has little effect on the ATPase activity, but reduces transport by 20 - 50%. Membrane can be kept on ice for 6 - 12 h without any serious deterioration.

2.2.9 Proton transport

In living cell H^+-ATPase functions in the biological energy conversion. It can convert the chemical free energy released by the hydrolysis of ATP into vectorial proton transport and may utilize the proton motive force (PMF) for ATP formation (Mitchell, 1970). According to chemiosmotic concept (Mitchell, 1976), the resulting pH gradient and membrane potential *i.e.* primary active transport may be used as the driving force to transport solutes and ions across the membrane (secondary active transport) (Leigh, 1983; Reinhold and Kaplan, 1984). Na^+ (Niemietz and Willenbrink, 1985; Garbarino and DuPont, 1988; Matoh et al., 1989), Ca^{2+} (Rasi-Caldagno et al., 1982; Schumaker and Sze, 1985, 1986; Joyce et al., 1988), sulfate (Cram, 1983), Cl^- and NO_3^- (Kaestner and Sze, 1987; Schumaker and Sze, 1987) are the most important ions which are transported through the tonoplast in the exchange of H^+. Active glucose transport via antiport with proton has also been reported by Guy et al. (1979), Thom and Komor (1984c), Rausch et al. (1987). Doll et al. (1979), Kaiser and Herber (1984), Briskin et al., (1985) have found that sucrose transport across the tonoplast against a concentration gradient is energized by ion-translocating ATPase activity. Willenbrink and Doll (1979) have found that sucrose transport into vacuole proceeds partly by an active transport and partly by passive permeation. Marin et al. (1981a), Oleski et al. (1987b) and Rentsch and Martinoia (1991) have found that citrate uptake is ATP dependant. Amino acids like malate (Luttge and Ball, 1979), phenylalanine (Homeyer and Schultz, 1988 and Homeyer et al., 1989) are accumulated into vacuoles coupled with proton translocation. Transport of anthocyanin (Hopp and Seitz, 1987) into vacuole by electrochemical potential gradient at tonoplast is also reported. Alkaloid like serpentine is

located inside the vacuole. Synthesis of alkaloids takes place in cytoplasm and the transport across the tonoplast into vacuole has been characterized as an active energy requiring mechanism (Deus-Neumann and Zenk, 1984). Jochem et al. (1984) have found that presence of Mg-ATP electrically polarizes tonoplast of the isolated vacuoles about 8.6 mV from +9.2 mV to +17.8 mV. John and Miller (1986) have observed that proton translocation by the ATPase results in a positive displacement of the membrane potential about 7 mV. The initial rate of proton pumping inside sealed right side out tonoplast vesicles as indication of ATPase activity is measured with fluorescent probe. Oxanol-V, AO, ACMA (9-amino-6-chloro-2-methoxy acridine), quinacrine are the fluorescent probes commonly used as indicator of proton gradients. There are ample evidences that the tonoplast H^+-ATPase catalyzes the electrogenic transport of H^+ across the vacuolar membrane of yeast (Kakinuma et al., 1981; Uchida et al., 1985), *Neurospora* (Bowman and Bowman, 1985) and higher plants (Poole, 1978; Bates et al., 1982; Sze, 1985; Blumwald and Poole, 1985; DuPont, 1987; Coyaud et al., 1987; Klaus et al., 1987; Pugin et al., 1991). Pumping activity of the tonoplast ATPase is also seen in latex of *Hevea* (Marin et al., 1981b) and *Tulipa* petal (Wagner and Lin, 1982). According to Nelson (1992) the tonoplast H^+-ATPase is one of the most fundamental proton pumps in nature.

2.2.10 Ultrastructure

The ATPase activity associated with the tonoplast has been detected cytochemically in tissue sections by employing the technique of lead precipitation of enzymically released organic phosphate in garden cress (Dorp et al., 1986) and *Kalanchoe daigremontiana* (Balsamo and Uribe, 1988)). In recent years ultrastructure of the ATPase particles associated with tonoplast vesicles has been demonstrated using negative staining.

Plant tonoplast ATPase is comprised of both peripheral and integral subunit similar to

F_0F_1 ATPase of mitochondria and chloroplast. Ultrastructure of the tonoplast ATPase has been examined by electron microscopy using negative staining in *Neurospora crassa* (Bowman et al., 1989) and also in higher plants like *Daucus carota* (Taiz and Taiz, 1990), *Mesembryanthemum crystallinum* (Klink and Luttge, 1991), soybean (Morre et al., 1991) and storage tissue of red beet (Getz and Klein, 1995). Ultramicroscopic structure of the tonoplast ATPase reveals that the dense enzyme particles associated with the tonoplast vesicles appear as typical head and stalk structure with a distinct apical cleft. The particles show a tendency to disappear when treated with nitrate plus Mg-ATP containing solution. Freeze-fracture electron microscopy has been used by Mariaux et al. (1994) to visualize intra-membranous particles corresponding to the tonoplast H^+-ATPase.

2.2.11 Interaction with plant hormones

Dohrmann et al. (1978) have reported the presence of three types of auxin binding sites in three different membrane fractions of maize coleoptile: site I in the endoplasmic reticulum, site II in the tonoplast and site III in the plasma membrane. Plasma membrane has shown to be the auxin transport system in close membrane vesicles (Hertel, 1983; Hertel et al., 1983; Benning, 1986; Heyn et al., 1987). Much of the information has not been reported about the role of site I and site II. Polar auxin transport is due to passive uptake of undissociated IAA across the plasma membrane (Rubery, 1979; Goldsmith and Goldsmith, 1981). According to Scherer (1984a), auxin stimulates H^+-extrusion by stimulation of a H^+-ATPase in *in vivo* and *in vitro*. The proton translocation may be either stimulated or inhibited by auxins, according to the hormone concentrations and the physiological condition of the plant (Santoni et al., 1990). Lesser amount of active carrier mediated symport of an auxin and proton is also found in tonoplast (Perbal and Driss-ecole, 1988). The role of phytohormone in H^+ extrusion by isolated

protoplast has been investigated by some workers. Cleland and Rayle (1975) have proposed that H^+, released by ATPase activity, triggers acid growth. In this context it is interesting that H^+ secretion has been shown to be increased by auxin (Cleland, 1975; Jacobs and Taiz, 1980), whereas in other cases this phytohormone does not significantly effect H^+ extrusion (Lado et al., 1976; Mettler and Leonard, 1979). Marre (1977) has found that the stomatal movement is due to interaction of IAA and ABA. ABA counteracts IAA by increasing membrane permeability resulting in loss of K^+ and thereby closure of stomata due to decrease in turgor. Schubert and Matzke (1985) have found that the turgor regulated growth of rape leaves can be controlled by an interplay of IAA and ABA which determines net H^+ secretion.

2.2.12 Interaction with phenolic compounds

It is not known whether tonoplast ATPase interacts with phenolic compounds or not, but it has been found that phenolic compounds are frequently accumulated as glycosides in vacuoles (Hosel, 1981). Glycoside formation increases solubility and mobility which facilitate storage and transport of the phenolic compounds. Accumulation of phenolic compounds have been described in vacuoles of horseradish root (Grob and Matile, 1980), grape berry (Moskowitz et al., 1981) and apple fruit (Yamaki, 1984). Alibert et al. (1982) have found that the uptake of o-coumaric acid glucosides by isolated vacuole of sweet clover is increased by Mg-ATP. The result suggests that the uptake is dependent on active transport mechanism. As the activity of tonoplast ATPase is Mg-ATP dependent, it may be possible that the enzyme plays a role in transporting phenolic compounds across the membrane.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Sodium orthovanadate, phloridzin, various nucleotides, acrylamide, bisacrylamide, Tris, SDS, *p*-NPP, asolectin (type IV) and molecular weight standards were obtained from Sigma Chemical Co., USA. Sepharose CL-6B was obtained from Pharmacia Fine Chemicals, Sweden. BCA protein assay reagent was obtained from Pierce Chemical Co. All other chemicals were obtained from Merck (India) or BDH (India) or SRL (India) and were analytical grade reagents. Carbon filmed grid was obtained from BIO-RAD (UK). Peanut (*Arachis hypogaea*) seeds were obtained from the local market.

3.2 METHODS

3.2.1 Germination of seeds

Seeds were surface sterilized with disinfectant, savlon for 15 min. Seeds were then thoroughly washed and were imbibed in tap water for 7 h. Imbibed seeds were grown on a moist, 4-layered pre-sterilized cheese-cloth in a plant growth chamber in dark at 30 ± 2°C and 80% relative humidity. Everyday the seeds were washed and non-germinated seeds were removed to avoid fungal infection. Seedlings were harvested at 7 d old stage of development for the isolation of membrane fractions.

3.2.2 Isolation of tonoplast membranes

Unless stated otherwise, all operations were carried out at 4°C. For isolation of tonoplast membranes, we followed the method of Màcri and Vianello (1987) with slight modifications. Following buffers were used:

Buffer-A: 20 mM HEPES-Tris buffer (pH-7.6) containing 250 mM sucrose, 1 mM EDTA, 10 mM $MgCl_2$ and 5 mM ß-mercaptoethanol.

Buffer-B: 10 mM HEPES-Tris buffer (pH-7.0) containing 125 mM sucrose and 5 mM ß-mercaptoethanol.

In brief, 50 g 7 d old etiolated seedlings were homogenized in a mortar using 200 ml buffer-A. The homogenate was filtered through an ordinary nylon sieve (pore diameter 500 μm approx.) and subsequently centrifuged at 13,000x g for 20 min using a fixed angle rotor (JA-20) in refrigerated high speed centrifuge (Beckman, model J2-21). The pellet was discarded and the supernatant was then centrifuged at 80,000x g for 30 min using a fixed angle rotor (type TFT 70.38) in a Centrikon, model T-2060 ultracentrifuge. This 13,000 - 80,000x g pellet constituted the microsomal fraction. The pellet was resuspended in 3.0 ml of buffer-B and layered over a 3-step sucrose gradient consisting of 3-layers of 15%, 35% and 45% sucrose (w/v) (from top to bottom) dissolved in buffer-B without 125 mM sucrose and ß-mercaptoethanol. This was centrifuged at 80,000x g for 2 h in a swing out rotor (type TST 41.14). Centrifugation resulted in separation of 3-membrane fractions, *i.e.* at sample / 15% sucrose, 15 / 35% sucrose and 35 / 45% sucrose interfaces which were carefully harvested using a Pasteur pipette. The membrane fraction collected at 15 / 35% sucrose interface constituted the tonoplast membranes (fig. 1).

3.2.3 ATPase assay

Solutions used in the assay are as follows:

Assay buffer: 50 mM Tris-MES, pH 8.0; 3 mM $MgSO_4$; 3 mM ATP; 50 mM KCl and 100 μM ammonium molybdate.

Standard solution: 10 mM KH_2PO_4, diluted 1:10 to obtain 1 mM.

Stopping reagent: 10% SDS.

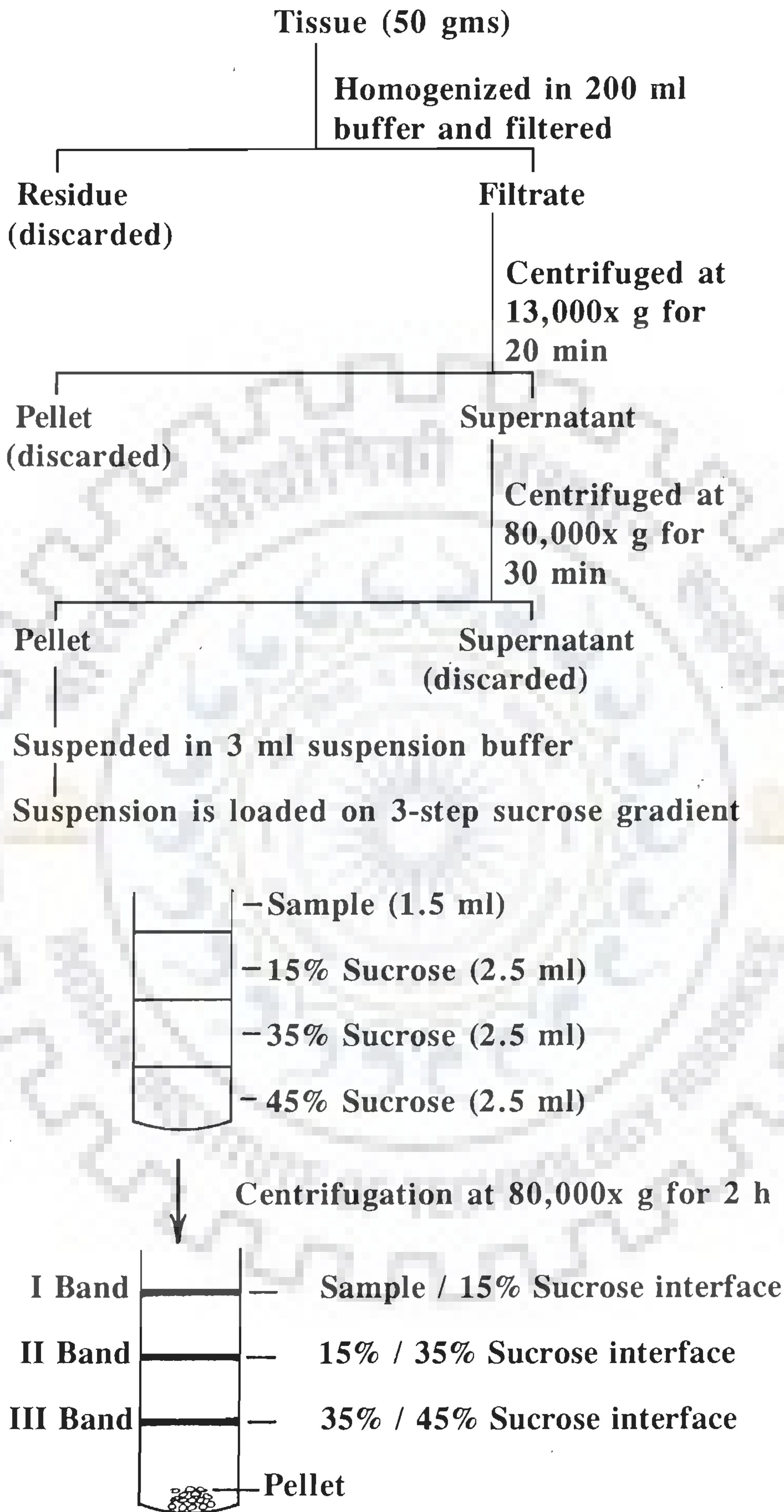

Fig. 1 **Isolation of tonoplast ATPase by differential and density gradient centrifugation.**

Colour reagent: 6 parts of 3.6 mM ammonium molybdate in 0.5 M H_2SO_4 + 1 part 10% ascorbic acid.

Assay procedure:

The tonoplast ATPase was analyzed by monitoring the release of Pi in the presence and absence of 50 mM NO_3^- ions. Inhibitors of other ATPases i.e. azide (0.5 mM) for mitochondria and vanadate (0.2 mM) for plasmalemma were routinely added in the reaction mixture. 100 μM ammonium molybdate was added to assay medium to inhibit acid phosphatase activity (Leigh and Walker, 1980a). The reaction was started by addition of membrane fractions (0.1 ml with about 2.0 - 2.5 μg protein) to reaction medium (0.45 ml) as follows:

	Probe	Standard	Blank
Assay buffer	300 μl	300 μl	300 μl
Water	50 μl	25 μl	50 μl
Standard solution	0 μl	25 μl	0 μl
Membrane extract	100 μl	100 μl	100 μl

The reaction mixture was immediately vortexed and incubated for 45 min at 37°C. The reaction was terminated by the addition of 10% SDS (0.5 ml). Parallel standards (with Pi) and blanks where 10% SDS was added prior to the addition of membrane fractions were run simultaneously. The amount of Pi released was determined after Leigh and Walker (1980b). In brief, colour reagent (0.6 ml) was added in the probe as well as blank and standard and incubation was carried out at 37°C for 45 min. After incubation, the reaction tubes were kept on ice for 5 min and the absorbance was recorded at 820 nm.

Triton solubilized enzyme was assayed as above except in presence of 0.2 mg phospholipid / ml reaction mixture. For the assay of gel filtered Triton solubilized enzyme.

concentration of phospholipid was reduced to 0.05 mg / ml.

3.2.4 Treatment with detergents

Detergents like Triton X-100, Brij-35, sodium deoxycholate and CHAPS were tested to see the effect of detergent on membrane bound enzyme. During this experiment several concentrations of each detergent were added separately in the assay medium. For Triton X-100 and Brij-35, concentrations were 0.1, 0.4, 0.7 and 1.0%. However 0.05, 0.1, 0.2 and 0.3% concentrations were used for sodium deoxycholate and CHAPS.

3.2.5 Solubilization of tonoplast ATPase

For solubilization of the tonoplast ATPase, methods proposed by Randall and Sze (1987a) was followed. One volume of ice cold solubilization buffer (20 mM HEPES-Tris buffer (pH-7.4) containing 10% Triton X-100, 60% (v/v) glycerol and 0.3 mM ATP) was mixed with an equal volume of tonoplast membranes (2 mg protein / ml membrane fraction). Final detergent concentration was 5%. The solution was allowed to stand on ice for 30 min and subsequently centrifuged at 105,000x g for 1 h in a TFT 70.38 rotor. The supernatant constituted the crude solubilized tonoplast ATPase preparation. To determine the optimum concentration of the detergent, different concentrations of Triton X-100 were added separately to the solubilization buffer in similar way as stated above. Final detergent concentrations were 1, 2, 3, 4, 5 and 6%.

3.2.6 Phospholipid preparation

Phospholipid asolectin (Sigma type IV) was prepared according to Manolson et al. (1985). 25 mg phospholipid was dissolved in 2 ml chloroform in a vial and a thin film of phospholipid was formed on the side of the vial by the evaporation of chloroform under nitrogen gas. Residual solvent was removed by lyophilization for 3 h, the vial was closed under N_2 and stored at -80°C. 25 mg / ml stock suspension was prepared in 20 mM HEPES-Tris (pH-7.4) and

sonicated in a bath sonicator for 20 min at 10°C immediately before use. From the stock solution different dilutions were made according to requirement.

3.2.7 Determination of optimum phospholipid concentration, used for recovery of tonoplast ATPase, for different concentration of Triton X-100

To see the effect of Triton X-100 and phospholipid concentration on solubilized tonoplast ATPase activity, the membrane fraction-II was solubilized at 5% (v/v) Triton X-100 and then diluted to 0.005% Triton X-100. Increasing amount of Triton X-100 was added to assay mixture to obtain various final Triton X-100 concentrations like 0.01, 0.025, 0.05 and 0.1%.

3.2.8 Gel filtration chromatography

3.2.8.1 Elution buffer

The elution buffer used for gel filtration chromatography had been prepared by the methods proposed by Randall and Sze (1987a) and was 20 mM HEPES-Tris buffer (pH-7.4) containing 0.2% Triton X-100, 30% (v/v) glycerol, 0.15 mM ATP and 0.1 mg phospholipid / ml.

3.2.8.2 Gel filtration

Fine particles were removed by decantation from swollen Sepharose CL-6B gel obtained from Pharmacia Fine Chemicals in ready to use form as suspension in distilled water. Slurry was too thick to be poured into a column and was diluted with eluent to the required consistency. The gel was degassed and was poured into the vertically mounted column (1.5 x 55 cm) using a glass rod. The eluent was also degassed. Two column volumes of eluent were passed through the column in order to stabilize the bed and equilibrated with eluent buffer in flow rate of 9 ml / h. The column was run at 4°C. Homogeneity of the packed bed was checked by running Blue Dextran 2000 at a concentration of 2 mg / ml.

The solubilized enzyme (1.0 ml) was loaded on the column. The flow rate was

maintained at 7.5 ml / h and 0.5 ml fractions were collected for enzyme activity determination.

3.2.9 Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (1970). 7.5-20% gradient gel (73 x 83 x 0.75 mm) with 10 wells was prepared using LKB 2050 Midget Electrophoresis unit.

3.2.9.1 Preparation of separating and stacking gels

Various solutions used are as follows:

Solution-A: Acrylamide solution - 30% (w/v) acrylamide solution containing 0.8% (w/v) N, N'- methylene-bis-acrylamide.

Solution-B: Resolving gel solution - 1.5 M Tris-HCl buffer (pH-8.8) containing 0.7% (w/v) SDS

Solution-C: Stacking gel solution - 0.5 M Tris-HCl buffer (pH-6.8) containing 0.7% (w/v) SDS

Solution-D: 3.3% (w/v) ammonium persulfate. This solution was prepared fresh before use.

Separating gel (recipe for preparation of five 0.75 mm thick 7.5-20% gradient gel)

	Dense gel solution	Light gel solution
Solution-A	7.97 ml	2.96 ml
Solution-B	2.96 ml	2.96 ml
Water	0.595 ml	5.95 ml
Glycerol	0.296 ml	--
Solution-D	71.4 μl	71.4 μl
TEMED	5.95 μl	5.95 μl

Stacking gel (5%)

Solution A	1.5 ml
Solution C	2.5 ml
Water	6.0 ml
Solution-D	90 μl
TEMED	10 μl

3.2.9.2 Casting of gel

Gel was casted in a sandwich form using aluminum oxide plate together with the glass plate. Two spacers (0.75 mm) were placed between the glass plate and the aluminum oxide plate to form gel sandwich of uniform thickness. Five sandwiches were assembled with a plastic sheet in between two sandwiches to ensure the easy separation of the two sandwiches after polymerization; they were then stacked in the multicast frame. Gradient gels were poured through the bottom of the multiple casting chamber from the gradient maker. Gradient maker consists of two chambers - reservoir and mixing chamber. Reservoir chamber contained the heavy solution whereas the light solution was placed in the mixing chamber. In mixing chamber gradient mixing was started and then the mixed solution was pumped to the casting chamber by peristaltic pump. After casting the resolving gel, it was overlaid with water saturated isobutanol. Gel was allowed to polymerize for several hours. When polymerization was completed, isobutanol overlayer was rinsed off with distilled water. Stacking gel was added to each individual gel sandwich by Pasteur pipette. When all sandwiches had been filled, the combs were inserted in sequence. Gel was allowed to polymerize for at least one hour. Gels were removed and either stored in cold room or used for loading sample.

3.2.9.3 Sample preparation

Enzyme samples collected from different steps of purification were concentrated by precipitating the protein with 7.5% TCA followed by incubation in ice for 30 min and centrifugation at 10,000x g for 5 min. The pelleted protein was washed with 100% cold acetone. Residue, obtained by evaporating acetone in vacuum, was resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% ß-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue) followed by heating in a boiling water bath for 2-3 min. Standard proteins for molecular mass determination were also treated in a similar manner. After heating, the sample was allowed to cool to room temperature.

3.2.9.4 Electrophoresis

Using a microsyringe, 25 μl sample (10-20 μg protein) was underlayered through the electrophoresis buffer (25 mM Tris, 0.192 M glycine and 0.1% SDS, pH 8.3). The sample was run at 100 volt through stacking gel. Once the sample was concentrated at the interface of the stacking and separating gel, the current was increased to 120 volt and the electrophoresis was continued until the tracking dye reached at the bottom of the gel. After the run, the gel was removed and stained in a solution containing 25 ml acetic acid, 113 ml methanol, 0.2 g Coomassie-R-250 Brilliant Blue, made to 250 ml with water and kept for 1 h at room temperature. Protein was destained in destaining solution, acetic acid-methanol-water (10:25:65, v/v/v).

3.2.10 Determination of molecular weight

Lambin (1978) observed that there is an excellent linear relationship between the log of molecular weight (Mr) and the log of gel concentration (%T) and the correlation between them was significantly better than the one between log (Mr) and log of relative mobility. Distances

travelled by polypeptides were converted to gel concentrations (%T). Log Mr was plotted against the log %T for the standards using double log graph paper (fig. 2). Molecular weight marker used was Haemoglobin, cross-linked (bovine) (approximate molecular weight 16,000 monomer, 32,000 dimer, 48,000 trimer and 64,000 tetramer).

3.2.11 Preparation of Tris-MES buffer of different pH

Tris-MES buffer of different pH (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) were prepared to determine the pH optima.

3.2.12 Determination of K_m value

To determine K_m value, different concentrations of ATP (0.05, 0.1, 0.2, 0.3, 0.4, 0.7 and 1 mM) were used in assay buffer.

3.2.13 Treatment with DDT, EDTA and ß-mercaptoethanol

To see the effect of DDT, EDTA and ß-mercaptoethanol on tonoplast ATPase, these reagents were added in assay medium. For DDT, different concentrations like 1, 3, 5 and 10 mM were tested. Similarly 3 different concentrations (5, 10 and 15 mM) of EDTA and 4 different concentrations of ß-mercaptoethanol (5, 10, 15 and 30 mM) were used during assay.

3.2.14 Treatment with divalent cations

To determine the effect of different divalent cations on tonoplast ATPase activity 3 mM $MgSO_4$ was substituted by the same amount of $MnSO_4$, $ZnSO_4$ and $CaSO_4$ which were applied separately in assay medium.

3.2.15 Treatment with different nucleoside phosphates

Various nucleoside phosphates *i.e.* GTP, CTP, UTP, ADP, GDP, AMP, GMP, CMP and *p*-NPP at a concentration of 3 mM were applied separately in place of ATP in assay mixture to determine the true substrate for ATPase.

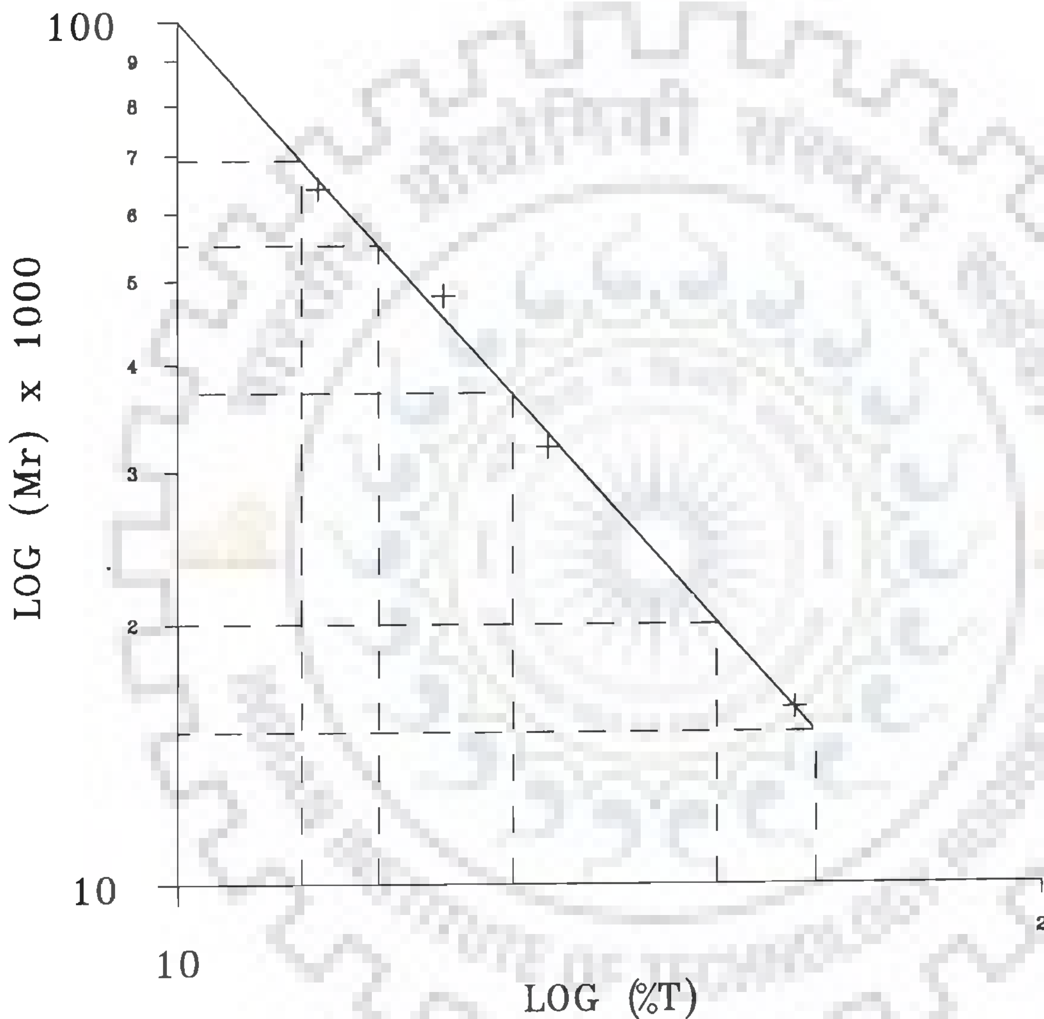

Fig. 2 **Determination of molecular weight of the purified tonoplast ATPase by SDS-PAGE.**

Relative mobility is converted to gel concentration (%T) and log (T%) is plotted against log of molecular weight (Mr). Molecular marker used is Haemoglobin, cross-linked (bovine) (approx. mol. wt. 16,000 monomer, 32,000 dimer, 48,000 trimer and 64,000 tetramer).

3.2.16 Treatment with monovalent cations

Monovalent cations like NH^{4+}, Na^+, Li^+, Cs^+ and Rb^+ were added separately in assay medium as chloride salts (50 mM) in place of 50 mM KCl.

3.2.17 Treatment with monovalent inorganic anions

50 mM of KBr, KI and $KClO_3$ were used separately in assay medium in place of KCl during experiments for determining the effect of monovalent inorganic anions.

3.2.18 Treatment with monovalent organic anions

50 mM inorganic anions (KCl) was replaced separately by same concentration of K-citrate and K-tartarate in assay medium.

3.2.19 Proton transport assay

Proton transport assay was done according to Pugin et al. (1991). ATP dependent proton pumping was measured by the initial rate of fluorescence quenching of the dye AO. The assay mixture contained 3 mM Tris-MES buffer (pH 8.0), 0.25 M sucrose, 3 mM $MgSO_4$, 50 mM KCl, 5 μM AO, 3 mM ATP, tonoplast protein (40-50 μg/ml). Fluorescence quenching of the dye was monitored with a spectrofluorimeter (model SLM Aminco 8000C, made in USA) with excitation and emission wavelengths of 495 nm and 520 nm respectively. The initial rate was determined in the first two minutes following the addition of 3 mM ATP. When a steady state of pH gradient was formed, it was discharged by addition of 2 μl of saturated NH_4Cl. The initial rate of quenching was given by $(\Delta F/F)x100\ min^{-1}\ mg^{-1}$ protein, F is the fluorescence recovered after the addition of NH_4Cl.

To see the effect of nitrate, 50 mM KNO_3 was added to the assay mixture both before the initiation of the reaction by ATP and after steady state was achieved.

3.2.20 Treatment with plant hormones

Different plant hormones like auxins (IAA, IBA, NAA and PAA), kinetin, GA_3 and ABA were tested to see the effect of phytohormones on tonoplast ATPase. Stock solutions of IAA, IBA, NAA, PAA, kinetin and ABA were prepared in 1N NaOH solution and GA_3 was dissolved in 50% ethylalcohol. In *in vitro* experiment three different concentrations (10^{-3}, 10^{-4} and 10^{-5} M) of each hormone solution were added separately to assay medium and incubated for 45 min. After incubation ATP was added to start the reaction and assay was performed as described in assay procedure. During assay control was run both in presence of appropriate concentration of NaOH and ethylalcohol separately. In *in vivo* experiment, the seeds were treated with above mentioned phytohormones of three different concentrations (10^{-3}, 10^{-4} and 10^{-5} M) separately. In brief, the seeds were imbibed for 24 h with appropriate hormone solution and then grown in plant growth chamber as described in section **3.2.1**. After seven day, tonoplast membranes were isolated and ATPase assay was done as described before.

3.2.21 Treatment with phenolic compounds

Different phenolic compounds like hydroquinones, hydroxybenzoic acid, chlorogenic acid and cinnamic acid were tested to see the effect of phenolic compounds on tonoplast ATPase. Amongst phenolic compounds hydroquinone and chlorogenic acid were dissolved in distilled water but hydroxybenzoic acid was dissolved in 1N NaOH and cinnamic acid was dissolved in methanol. Three different concentrations (10^{-3}, 10^{-4} and 10^{-5} M) of phenolic compounds were used during both *in vivo* and *in vitro* experiments. At the time of *in vitro* experiment the appropriate phenolic solution was added directly to the assay medium excepting cinnamic acid. However 0.1% Triton X-100 was added to the cinnamic acid before adding assay buffer. Three separate controls were run using distilled water, appropriate concentration of NaOH and

methanol mixed with Triton X-100 (in appropriate conentration).

During *in vivo* experiment the seeds were imbibed in appropriate phenolic solution for 24 h before they were allowed to germinate in growth chamber in a similar manner as was done in case of *in vivo* experiment with phytohormones.

3.2.22 Isolation of membrane fraction of mitochondria

Mitochondrial membrane fractions were isolated according to Nagahashi and Hiraike (1982). Seedlings were homogenized by using the method followed for the isolation of the tonoplast membrane. Homogenate was centrifuged at 1,000x g for 10 min. Pellet was discarded and the supernatant was centrifuged at 6,000x g for 10 min. The pellet consisting of membrane fraction of mitochondria was suspended in suspension buffer (buffer-B, as described in **3.2.2**).

3.2.23 Electron Microscopy

Vacuolar and mitochondrial membranes immediately after isolation were diluted to protein concentrations of 0.5 mg - 1 mg / ml in ATPase reaction mixture (Tris-MES, pH-8.0, 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate). To some samples, KNO_3, azide and vanadate were added separately to a final concentration of 100 mM, 0.5 mM and 0.2 mM respectively and the samples were incubated for 20 - 30 min. 2 μl sample was applied to carbon filmed (thickness 10 nm) grids (G 200). After 1 min the membrane solution was drawn off by absorbent paper and the remaining membranes attached to the grid were immediately negatively stained for 1 min with aqueous solution of 2 μl 1% PTA, pH-6.8 (adjusted with KOH). The excess solution was slowly drawn off using absorbent paper. The grid was briefly air dried and observed under transmission electron microscope (Phillips EM-400) at primary magnification of 45,000X at an accelerating voltage of 80 kV. All operations were done at room temperature (about 20°C).

3.2.24 Protein determination

Protein in various samples was determined by either the method of Lowry et al. (1951) or by Pierce BCA protein assay reagent (Smith et al., 1985) using bovine serum albumin as a standard.

Estimation of protein by the method of Lowry

Principle: The final colour is developed as a result of

i) Biuret reaction of protein with copper ions in alkali.

ii) Reduction of phosphotungstic phosphomolybdic reagent with tyrosine and tryptophan present in the treated protein.

Reagents:

Reagent A:	2% sodium carbonate in 0.1N sodium hydroxide
Reagent B:	0.5% copper sulphate pentahydrate in 1% potassium sodium tartarate. Prepared fresh before use.
Reagent C:	Alkaline copper reagent comprising 50 parts of reagent A and 1 part of reagent B.
Reagent D:	Folin Ciocalteau reagent, diluted 1:1 with glass distilled water.

Assay procedure:

1. Serial dilutions of 20 to 200 μg/ml were made from a stock solution of 1 mg/ml Bovine Serum Albumin.
2. Aliquots containing suitable amount of protein were made upto 1 ml in 0.1N sodium hydroxide.
3. To this 1 ml of reagent C was added and mixed.
4. The mixture was then incubated for 15 minutes at room temperature.

5. 0.1 ml of reagent D was added and mixed immediately.
6. The absorbance at 690 nm was recorded on a Beckman DU-6 spectrophotometer after 45 minutes incubation at room temperature, using a reagent blank.

Estimation of protein with the Pierce BCA reagent

Principle: BCA protein assay reagent combines the well known biuret reaction with the unique features of BCA. The purple reaction product formed by the interaction of two molecules of BCA with one cuprous ion (Cu^{1+}) is water soluble and exhibits a strong absorbance at 562 nm. This allows the spectrophotometric quantification of protein in aqueous solutions.

Reagents:

BCA protein assay reagent was a pre-formulated reagent system that was supplied in two separate bottles.

Reagent A: 1000 ml base reagent which contains sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartarate in 0.2N NaOH.

Reagent B: 25 ml of 4% copper sulphate solution

Working reagent: 50 parts of reagent A was mixed with 1 part of reagent B.

Assay Procedure

1. Different protein standards were prepared from the stock solution of 2 mg/ml BSA.
2. 0.1 ml of each standard or unknown protein sample was pipetted into appropriately labeled test tubes. For blank, 0.1 ml diluent was used.
3. 2.0 ml of working reagent was added to each tube and mixed well.
4. All tubes were incubated at 37°C for 30 min.
5. After incubation all tubes were cooled to room temperature.
6. Absorbance was noted at 562 nm.

CHAPTER 4

4.0 RESULTS

4.1 Isolation of tonoplast membrane fraction

Plant tissue is homogenized and microsomal pellet is obtained from plant extract through different steps of centrifugation as described in methods (**3.2.2**). Centrifugation of an 80,000x g pellet,obtained from 7 d old peanut seedlings,on a 3 step sucrose gradient results in separation of 3 membrane fractions, i.e. at sample/15% sucrose, 15/35% sucrose (densities, 1.0622/1.1566 g/cm^3) and 35/45% sucrose (densities, 1.1566/1.2090 g/cm^3) interfaces which are referred as fraction I, II and III respectively (fig. 1). In addition, a fourth fraction chiefly made up of fat droplets (observed under light microscope) floats at the top of sample zone. On the basis of known densities (1.10 - 1.12 g/cm^3) of tonoplast membranes (Quail, 1979), fraction II (densities, 1.0622/1.1566 g/cm^3) is likely to be enriched in them.

4.2 Inhibitor sensitivity characteristics of ATPases bound to different membrane fractions

The results on inhibitor sensitivity characteristics of the ATPases associated with various fractions are given in table 1. Phloridzin is ineffective as an inhibitor of ATPases bound to various fractions. Addition of 0.5 mM azide, a mitochondrial ATPase inhibitor causes inhibition of ATPases of all fractions to varying degrees which does not exceed 10%. Vanadate causes 41% and 6% inhibition of fraction III and fraction II associated ATPases respectively. ATPase associated with fraction I is not inhibited by vanadate. Since vanadate sensitive ATPase is bound to plasmalemma it appears that fraction III is enriched in these types of membranes. Addition of 50 mM NO_3^- ions which are specific inhibitors of tonoplast ATPase activity, causes significant inhibition (45%) of the ATPase bound to fraction II.

Table 1. **Effect of various inhibitors on ATPase activity associated with various membrane fractions**

Inhibitor type	Fraction I		FractionII		Fraction III	
	A	B	A	B	A	B
Control[1]	43.0	100	17.5	100	13.3	100
Phloridzin, 1 mM	45.15	105	17.5	100	13.3	100
Vanadate, 0.2 mM	43.0	100	16.45	94	7.85	59
Azide, 0.5 mM	41.71	97	15.92	91	12.77	96
Nitrate, 50 mM	43.0	100	9.62	55	12.5	94

1 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate

A Specific activity (μmol Pi mg^{-1} protein h^{-1})

B . % ATPase activity

Thus on the basis of the density range of fraction II which coincides with that of tonoplast membranes and the fact that the associated ATPase is sensitive to NO_3^- ions, it can be said that this fraction is enriched in tonoplast membranes. Further as evident from the extent of inhibition caused by other inhibitors, this fraction does not appear to be significantly contaminated by other membrane fractions. This fraction was used for solubilization, partial purification and study of characteristics of tonoplast ATPase.

4.3 Distribution of tonoplast ATPase activity in various fractions obtained during centrifugation

Table 2 shows the distribution of total and NO_3^- sensitive ATPase activity in various fractions obtained after centrifugation. Centrifugation of cell homogenate at 10,000x g has resulted in two fractions i.e. 10,000x g pellet and 10,000x g supernatant. Nearly all of NO_3 sensitive ATPase and 88% of total ATPase activity is recovered in 10,000x g supernatant. In the next step 10,000x g supernatant has been centrifuged at 80,000x g. This has resulted in pelleting of various membranes *i.e.* the microsomal membranes and a part of mitochondrial and chloroplast membranes. The 80,000x g pellet contains 70% and 89.4% of the total and the NO_3^- sensitive ATPase activity respectively. The supernatant which contained only the little amount of NO_3^- sensitive activity was not used further for isolation of tonoplast membrane enriched fraction. Microsomal pellet (*i.e.* 80,000x g pellet) is resuspended and is loaded on 3 step density gradient as described in methods. This has resulted separation of three membrane fractions as already described. Whereas only about half of the total ATPase activity is found to be associated with membrane fraction II, about 82% of NO_3^- sensitive activity is recovered from this fraction. No NO_3^- sensitive ATPase activity is detected in membrane fraction I. The third fraction also contains only very minor amount of NO_3^- sensitive activity.

Table 2. **Distribution of tonoplast ATPase activity in various fractions obtained during differential and step-gradient centrifugation.**

Fraction	Total ATPase activity*	% total ATPase activity recovered	ATPase activity* in presence of NO_3^-	NO_3^- sensitive ATPase activity*	% NO_3^- sensitive activity recovered
10,000x g pellet	5.7	11.9	5.59	0.11	0.8
10,000x g superna-tant	42.3	88.1	28.22	14.08	99.2
80,000x g pellet	33.4	70.0	20.71	12.69	89.4
80,000x g superna-tant	10.9	22.7	9.27	1.64	11.5
Membrane frac-tion I	2.1	4.4	2.15	0	0
Membrane frac-tion II	24.3	50.6	12.69	11.62	81.8
Membrane frac-tion III	8.5	17.7	7.65	0.85	6.0

* ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}

Assay buffer - 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate

4.4 Changes in ATPase activity accompanying seedling development

Fig. 3 shows that the activity of ATPase is very less in first 3 days. Significant increase of activity is noted from 4th day. The enzyme activity of 4 d old seedlings is 6.96 μmol Pi mg^{-1} h^{-1} which is about 7 fold higher than that of 1 d old seedlings. The activity peak is reached on 7th day. However, the activity starts decreasing from 8th day onwards and on 10th day ATPase activity is less than half than that of 7th day.

4.5 Distribution of ATPase activity in different parts of a seedling

It is noted that the amount of ATPase activity (as determined in terms of specific activity, activity per organ and activity per g. of tissue) is not uniform in different parts of a seedling (fig. 4). It is clearly demonstrated in the figure that hypocotyl shows maximum activity compared to all other organs of the seedlings (specific activity - 16.57 μmol Pi mg^{-1} protein h^{-1}; activity per organ - 7.36 μmol Pi h^{-1}; activity per g of tissue - 221.43 μmol Pi h^{-1}). However, relative activities of the organs other than hypocotyl, vary when measured in different units. For example, the specific activity of root (4.405 μmol Pi mg^{-1} protein h^{-1}) is more compared to cotyledon (0.825 μmol Pi mg^{-1} protein h^{-1}) and epicotyl (0.64 μmol Pi mg^{-1} protein h^{-1}). But in case of activity per organ and activity per g of tissue cotyledon shows maximum activity compared to root and epicotyl. Epicotyl shows minimum activity when measured in terms of specific activity and activity per organ. However, when activity is expressed in terms of per g of tissue, root shows least activity.

Could you discuss the probable causes of this difference?

4.6 Properties of tonoplast ATPase

4.6.1 Optimum pH

A very sharp pH optima for the NO_3^- sensitive ATPase is obtained at a pH value of 8.0 (fig. 5). At this pH, ATPase activity is 22.3 μmol Pi mg^{-1} protein h^{-1}. At pH 7.5 about 72%

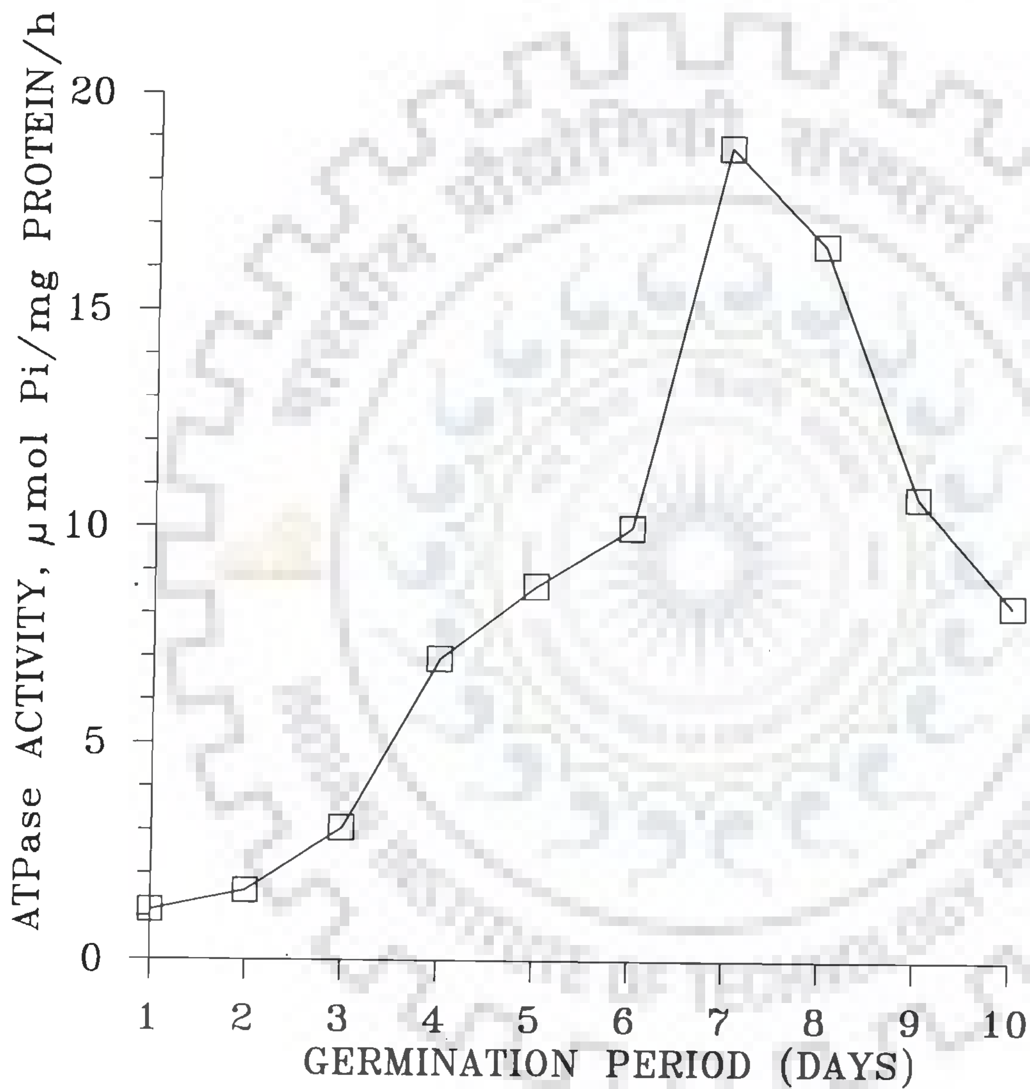

Fig. 3 ATPase activity as a function of germination period.

The enzyme activity was assayed after different germination period.

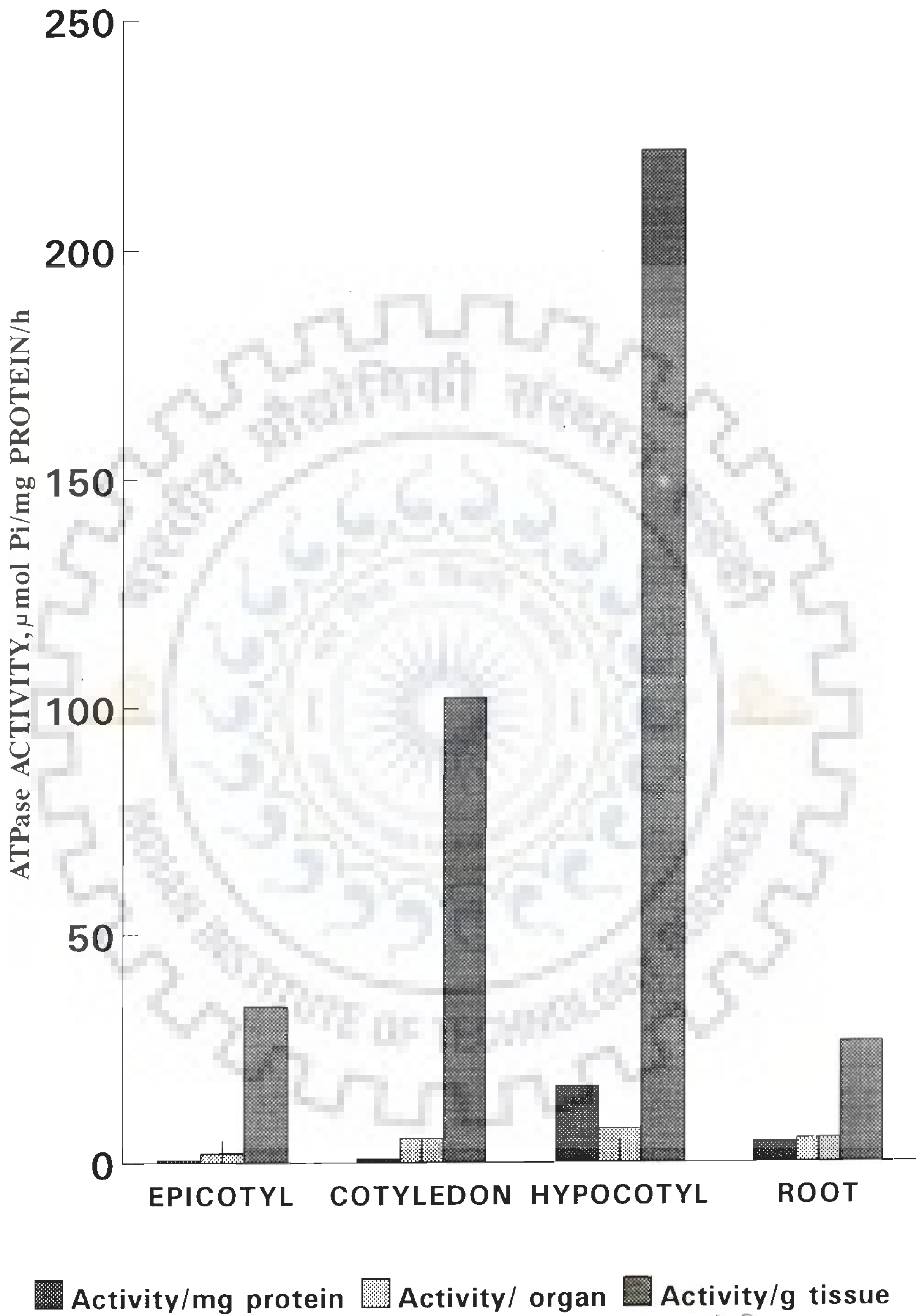

Fig. 4 **Distribution of tonoplast ATPase activity on different parts of the seedling.**

— age of seedling ?

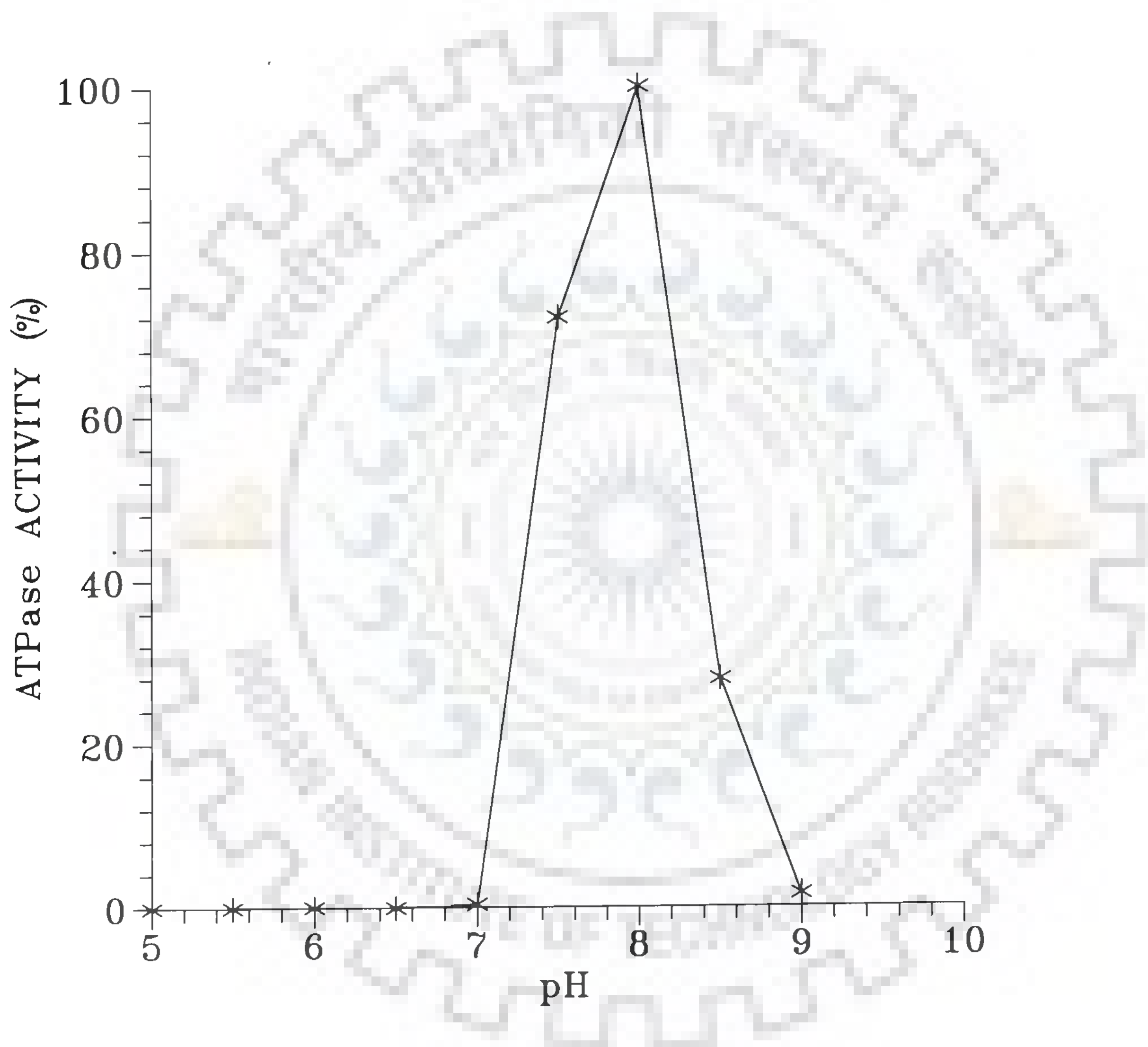

Fig. 5 pH optima of tonoplast ATPase activity.

The absolute value of 100% ATPase activity is 22.3 μmol Pi mg^{-1} protein h^{-1}. Tris-MES buffer of different pH (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) were used.

ATPase activity still remains. However at pH 7.0, 8.5 and 9.0, the ATPase activity is only 0.1%, 28% and 0.4% of the activity observed at pH optima. Nitrate sensitive ATPase activity could not be detected at pH values of 6.5 and <6.5.

4.6.2 K_m and V_{max} values

Fig. 6 shows the effect of substrate concentration on the velocity of the ATPase catalyzed reaction. As can be seen, the plot between velocity versus substrate concentration is hyperbolic and the double reciprocal plot of 1/v versus 1/[S] is also linear. These results indicate that the reaction kinetics follow the Michaelis-Menten equation. The straight line of Lineweaver-Burk plot has an intercept of $1/V_{max}$ on the 1/v axis, and an intercept of $-1/K_m$ on the 1/[S] axis. K_m and V_{max} values of the enzyme are determined by Lineweaver and Burk plot using Mg-ATP as the substrate and are found to be 0.15 mM and 3.1 μmol Pi released mg^{-1} protein h^{-1} respectively.

4.6.3 Effect of DTT, ß-mercaptoethanol and EDTA

Addition of both DTT and ß-mercaptoethanol to the assay medium has resulted in loss of ATPase activity (table 3). In presence of DTT at various concentrations i.e., 1, 3, 5 and 10 mM, the loss in activity is about 55%. At 10, 15 and 30 mM concentrations, ß-mercaptoethanol causes about 65% loss of activity, whereas at 5 mM concentration loss of activity is less (45%). In contrast addition EDTA results in much higher loss of ATPase activity. At all concentrations *i.e.* 5, 10 and 15 mM, EDTA causes about 90% decline in ATPase activity.

4.6.4 Requirement of divalent metal cations

It is evident from our data on divalent metal cation requirement (table 4) that these ions are necessary for the optimal enzyme activity. In presence of KCl, the order of divalent cation requirement for the enzyme is $Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}$. Compared to Mg^{2+} ions, Mn^{2+}

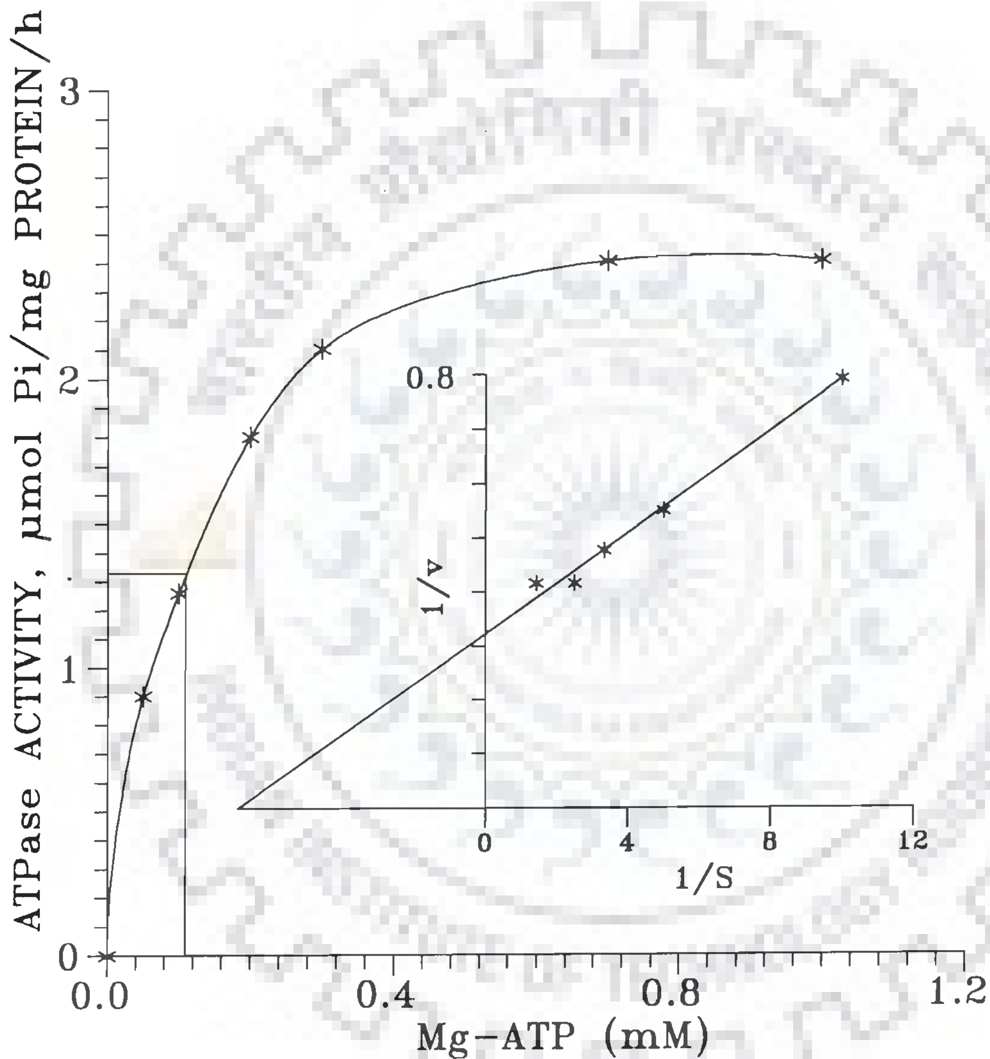

Fig. 6 Saturation plot and Lineweaver and Burk plot for determination of K_m and V_{max} values of tonoplast ATPase.

Average K_m and V_{max} estimated by Lineweaver and Burk plot were 0.15 mM and 3.1 μmol Pi mg^{-1} protein h^{-1} respectively.

Table 3. Effect of DTT, EDTA and ß-mercaptoethanol on tonoplast ATPase activity

Reagent	mM concentration	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity
DTT	0[1]	22.05	100.0
	1	9.59	43.5
	3	9.70	44.0
	5	9.92	45.0
	10	9.61	43.6
EDTA	0[1]	18.30	100.0
	5	2.36	12.9
	10	1.99	10.9
	15	1.78	9.75
ß-mercaptoethanol	0[1]	13.05	100.0
	5	8.55	65.5
	10	4.37	33.5
	15	4.57	35.0
	30	4.18	32.0

1 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate

Table 4. Effect of divalent cations on tonoplast ATPase activity

Divalent salt (3 mM)	ATPase activity in presence of KCl		ATPase activity in absence of KCl	
	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity with $MgSO_4$	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity with $MgSO_4$
0[1]	*	*	*	*
$MgSO_4$	3.0	100	1.1	100
$MnSO_4$	2.25	75	1.79	163
$ZnSO_4$	1.2	40	1.0	91
$CaSO_4$	0.6	20	0.41	37
1.5 mM $MgSO_4$ + 1.5 mM $CaSO_4$	1.05	35	2.64	240

1 50 mM Tris-MES buffer (pH 8.0), 3 mM ATP, 100 μM ammonium molybdate and 50 mM KCl (added only when KCl is present)

* Not detected

ions are about 75% effective in restoring ATPase activity. No activity could be detected in absence of divalent cations. In presence of Ca^{2+} ions alone, ATPase activity is only 20% of the control. When both Mg^{2+} and Ca^{2+} ions are combined in equimolar concentrations, tonoplast ATPase activity is only 35% than in the presence of Mg^{2+} ions alone. However, interestingly in absence of KCl, the order of divalent cation requirement is changed with Mn^{2+} ions being the most optimal. Then, when compared to Mg^{2+} ions alone, there is about 2.5 fold increase in tonoplast ATPase activity in absence of KCl when both Ca^{2+} and Mg^{2+} ions are present together.

4.6.5 Substrate specificity

Amongst various nucleoside phosphates tested, ATP is the most effective for the hydrolysis reaction (table 5). GTP is also partially hydrolysed. With GTP as substrate, the enzyme activity is 12.14 μmol Pi mg^{-1} protein h^{-1} which is nearly half of the activity observed in presence of ATP. Hydrolysis of other nucleoside phosphates except UTP (12% of the reaction in comparison with ATP) is less than 10%. In general, nucleoside monophosphates and diphosphates are hydrolysed to a much lesser degree than nucleoside triphosphates. *Para-*nitrophenyl phosphate is not hydrolysed at all.

4.6.6 Effect of monovalent inorganic anions

In absence of KCl tonoplast ATPase activity is 1.02 μmol Pi mg^{-1} protein h^{-1} (table 6). Addition of Cl^- ions causes about 3 fold enhancement in enzyme activity. In presence of Br^- ions activity is 77% than that of Cl^- ions. Activity further significantly declines to only 36% in presence of I^- ions. In contrast tonoplast ATPase activity is completely inhibited by ClO_3^- ions. Thus the order of effectiveness of inorganic monovalent anions is $Cl^- > Br^- > I^-$.

4.6.7 Effect of organic anions

The data on effect of organic anions on tonoplast ATPase activity is given in table 7.

Table 5. Tonoplast ATPase activity towards various nucleoside phosphates and *p*-nitrophenyl phosphate

Substrate (3 mM)	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity
ATP	23.80	100
GTP	12.14	51
CTP	1.90	8
UTP	2.86	12
ADP	0.71	3
GDP	1.79	7.5
AMP	0.24	1
GMP	1.07	4.5
CMP	1.07	4.5
p-NPP	0	0

Assay buffer - 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 50 mM KCl and 100 μM ammonium molybdate, substrate (3mM)

Table 6. Effect of monovalent anions on tonoplast ATPase activity

Monovalent salts (50 mM)	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity
0[1]	1.02	32
KCl	3.20	100
KBr	2.46	77
KI	1.15	36
$KClO_3$	0	0

1 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP and 100 μM ammonium molybdate

Table 7. Effect of organic anions on tonoplast ATPase activity

Salt (50 mM)	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity
KCl	12.60	100
K-Citrate	0	0
K-Tartarate	7.94	63

Assay buffer - 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP and 100 μM ammonium molybdate, salt (50 mM)

There is about 63% enzyme activity in presence of tartarate compared to the Cl^- ions control, whereas addition of 50 mM citrate results in complete inhibition of activity.

4.6.8 Effect of monovalent cations

Table 8 shows the effect of monovalent cations on tonoplast ATPase activity. With the exception of RbCl, addition of salts, causes about 3-4 fold stimulation in ATPase activity. The order of effectiveness of monovalent cations on the ATPase activity is NH_4Cl > KCl > NaCl > LiCl, CsCl > RbCl. In presence of NH_4Cl the ATPase activity is 24% higher than with KCl. Even in presence of RbCl, the enzyme activity is about 1.6 fold higher than the value obtained in the absence of salts.

4.6.9 Proton transport

Proton transport across the tonoplast vesicles was determined with AO as pH probe (fig. 7). Addition of ATP causes decrease in fluorescence which is linear for about 2 min, then reaches a steady state. Addition of NH_4Cl immediately reverses the quenching. NO^{3-} decreases (53%) the initial rate fluorescence quenching initiated by ATP (table 9). NO_3^- when added during steady state, initially quenching is increased and then proton gradient is dissipated.

4.6.10 Effect of various detergents on the activity of membrane bound tonoplast ATPase

For solubilizing the enzyme, effect of several detergents on the $N0_3^-$ sensitive ATPase activity was tested (table 10). Of the various detergents, sodium deoxycholate is ineffective as the enzyme activity is greatly reduced at 0.1 and 0.2% concentrations. Since these concentrations are much lower than the CMC of sodium deoxycholate (0.57%), micelle formation does not occur. In contrast, treatment of enzyme with Brij-35 and Triton X-100, at concentrations higher than their CMC values results in increase of activity. However with increase in concentration, the ATPase activity decreases in presence of Brij-35. On the contrary

Table 8. **Effect of monovalent cations on tonoplast ATPase activity**

Monovalent salts (50 mM)	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity
0[1]	1.39	32
KCl	2.95	100
NH_4Cl	3.66	124
NaCl	2.86	97
LiCl	2.60	88
CsCl	2.57	87
RbCl	1.53	52

1 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP and 100 μM ammonium molybdate

Table 9. **ATP dependent proton transport by tonoplast vesicles. Effect of nitrate on the initial rate of fluorescence quenching of the dye AO**

Treatment	Initial rate of fluorescence quenching [(ΔF/F) x 100 min^{-1} mg^{-1} protein]
0*	0
+ ATP	314 (100%)
+ ATP + KNO_3	146 (47%)

* 3 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 50 mM KCl, 0.25 M sucrose, 5 μM AO, tonoplast protein (40-50 μg/ml)

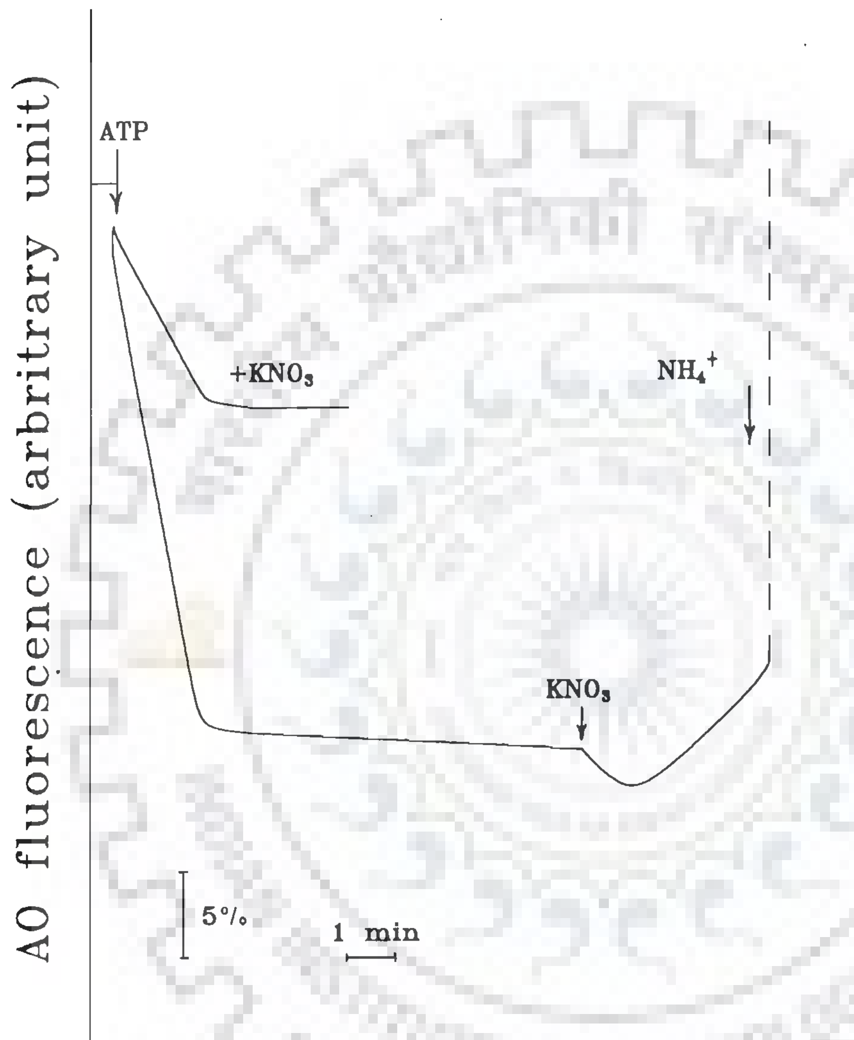

Fig. 7 **ATP dependent H^+ pumping by tonoplast vesicles and effect of NO_3^- on the same.**

H^+ pumping was measured by fluorescence quenching of acridine orange.

Table 10. Effect of different detergents on tonoplast ATPase activity

Detergent	% Concentration	CMC	Specific activity (μmol Pi mg^{-1} h^{-1})	% ATPase activity
Triton X-100	0.0[1]	(0.02%)	20.50	100
	0.1		25.22	123
	0.4		27.39	133.6
	0.7		28.50	139
	1.0		28.39	138.5
Brij-35	0.0[1]	(0.01%)	21.90	100
	0.1		41.80	191
	0.4		37.01	169
	0.7		30.66	140
	1.0		29.35	134
Sodium deoxycholate	0.0[1]	(0.57%)	21.90	100
	0.05		22.56	103
	0.1		12.70	58
	0.2		1.31	6
	0.3		0	0
CHAPS	0.0[1]	(0.5%)	22.20	100
	0.05		24.53	110.5
	0.1		29.53	133
	0.2		13.76	62
	0.3		6.48	29.2

1 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate

ATPase activity remains stable at higher concentrations of Triton X-100 such as 0.7 and 1%. Moreover, the CMC of Triton X-100 is very low (0.02%). CMC of CHAPS is 0.5% and at 0.2% and 0.3% concentrations, it greatly decreases the tonoplast ATPase activity. Thus Triton X-100 appears to be fairly suitable detergent for solubilization of tonoplast ATPase.

4.7 Solubilization

Different concentrations of Triton X-100 were tested to determine its optimum concentration required for the solubilization of the enzyme (fig. 8). For this experiment 1 ml of membrane fraction containing about 2 mg of protein was mixed with an equal volume of Triton X-100 of different concentrations (final concentrations were 1, 2, 3, 4, 5 and 6%) separately. Membrane fraction was solubilized following the procedure as described in Methods. After solubilization, total protein content and ATPase activity were determined both in pellet and supernatant for each concentration of Triton X-100. The percent solubilization was calculated assuming the sum of the activities in the supernatant and the pellet as 100%. It is found that at 4 and 5% of Triton X-100, maximum ATPase activity *i.e.* 93% of the total activity is solubilized. However, the maximum amount of protein *i.e.* 67% of the total protein is obtained at 5% Triton X-100. At lower concentrations of Triton X-100 *i.e.* 1, 2 and 3%, the percent ATPase solubilization is much less *i.e.* 19, 35, and 79% respectively. Considering the values of maximum solubilization of the ATPase activity and the total protein, 5% Triton X-100 was used for solubilizing the enzyme and to maximize the possibility of formation of only one protein per detergent-protein micelle.

4.8 Effect of phospholipids on enzyme activity

It is apparent from the fig. 9 and 10 that phospholipid is essential for the recovery of ATPase activity in presence of Triton X-100. The membrane fraction-II was solubilized at 5%

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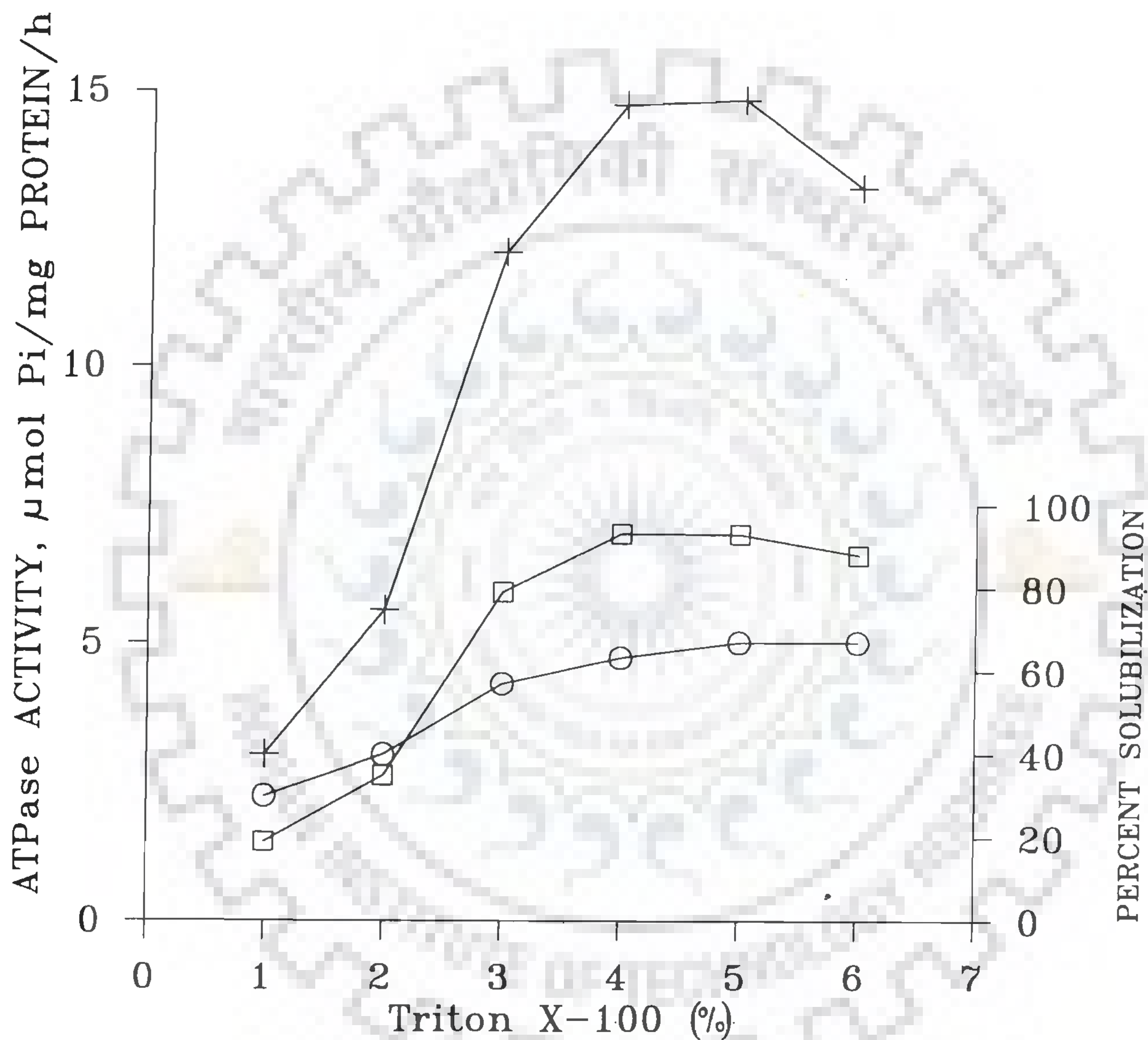


Fig. 8 **Solubilization of ATPase activity as a function of detergent concentration at a fixed protein concentration.**

The circle, square and plus signs denote percent solubilization of protein, percent solubilization of ATPase activity and specific activity of ATPase of solubilized enzyme respectively.

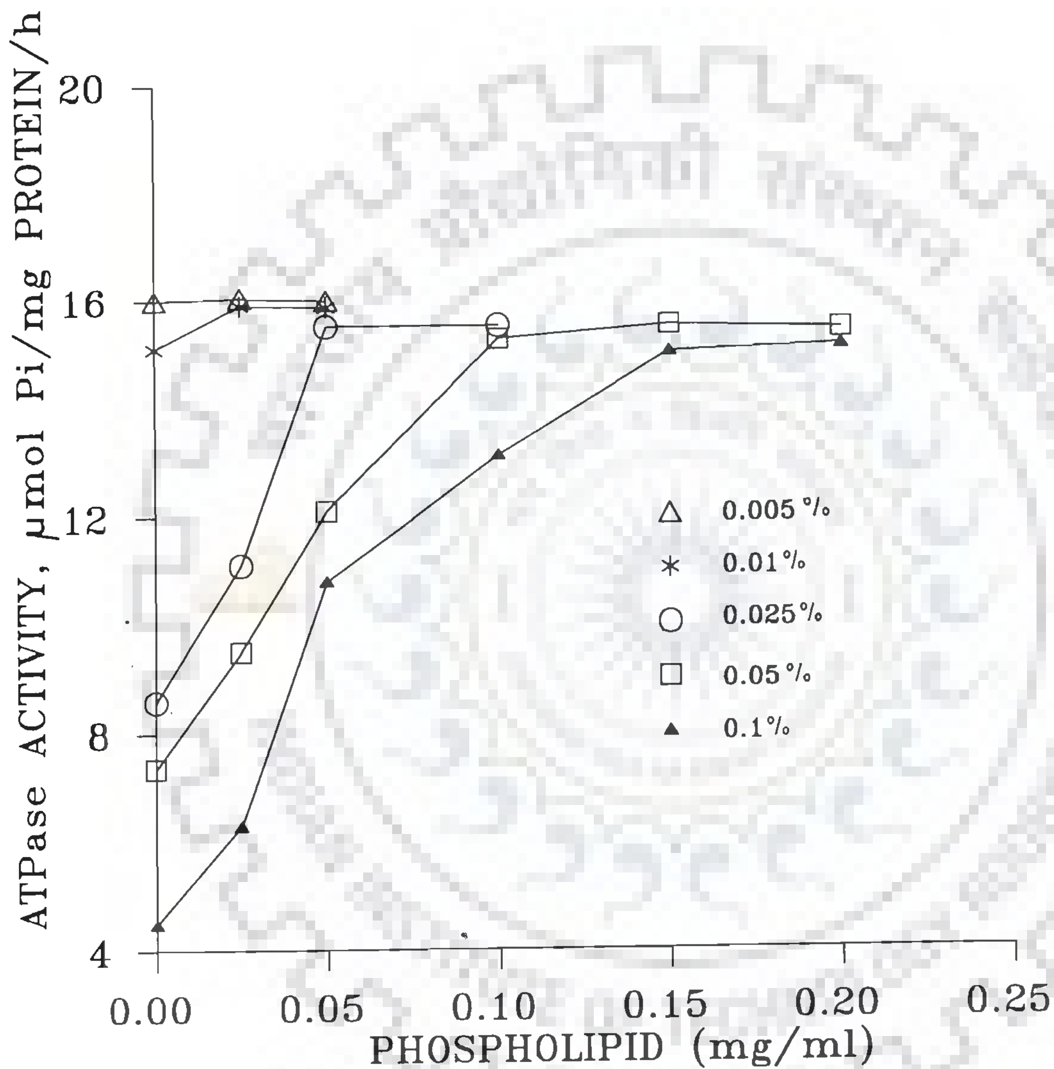


Fig. 9 Determination of optimum phospholipid concentration for restoration of ATPase activity in different concentrations of Triton X-100 preparations.

The different concentrations of Triton X-100 used are 0.005%, 0.01%, 0.025%, 0.05% and 0.1%. The symbols for corresponding concentrations are given in the figure legend.

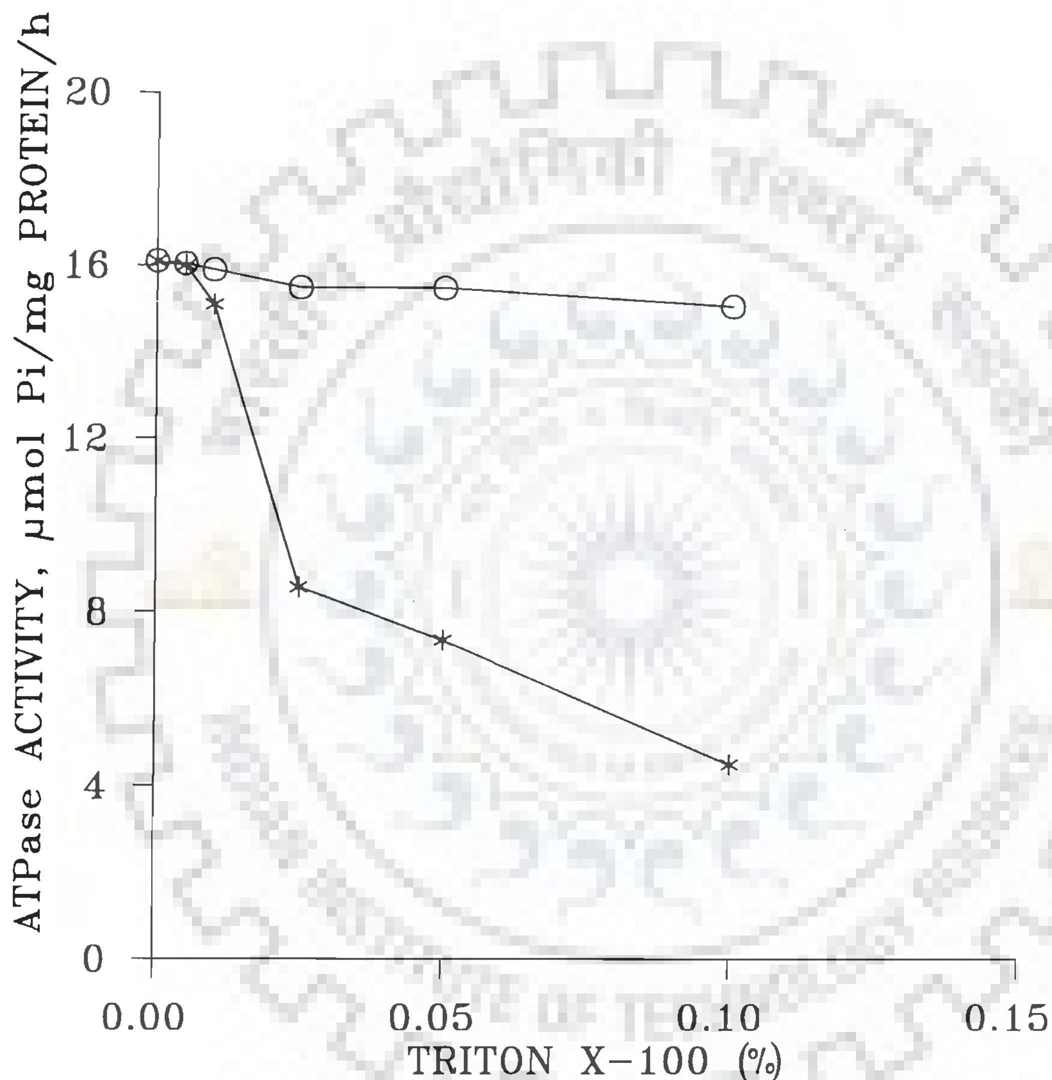


Fig. 10 Effect of Triton X-100 and phospholipid concentration on solubilized tonoplast ATPase activity.

ATPase activity is measured in the absence and presence of phospholipid. The membrane is solubilized at 5% (v/v) Triton X-100 and diluted to 0.1, 0.05, 0.025, 0.01 and 0.005% Triton X-100. The presence and absence of phospholipid are shown by circle and star symbols respectively.

(v/v) Triton X-100 and then diluted to 0.005, 0.01, 0.025, 0.05 and 0.1% Triton X-100. It is found that in the presence of 0.005% Triton X-100, ATPase activity is almost similar both in presence and absence of phospholipid *i.e.* ATPase activity is not stimulated by phospholipid. At increased concentrations of Triton X-100, phospholipid is effective in stimulating the enzyme activity. At 0.01% Triton X-100, enzyme activity has been increased in lesser degree in presence of phospholipid. At 0.025% Triton X-100 concentration, the ATPase activity is 8.58 μmol Pi mg^{-1} protein h^{-1} in the absence of phospholipid. When 0.025 and 0.05 mg/ml phospholipid was added separately to assay medium, ATPase activity is increased to 11.1 and 15.5 μmol Pi mg^{-1} protein h^{-1} respectively. Further increase in phospholipid concentration (0.1 mg/ml) does not cause an increase in ATPase activity. Thus in the presence of 0.025% Triton X-100, optimum phospholipid concentration required for the recovery of tonoplast ATPase activity is 0.05 mg/ml. Similarly it is found that at 0.05% and 0.1% Triton X-100, optimum phospholipid concentration for the recovery of ATPase activity is 0.15 and 0.2 mg/ml respectively.

During assay, enzyme extract was diluted at 1:100 with glass distilled water. So, 5% Triton X-100, used for solubilizing the enzyme, was diluted simultaneously with the enzyme to 0.05% Triton X-100. Thus 0.2 mg phospholipid/ml was added to the assay medium to recover the ATPase activity. Over 90% ATPase activity is recovered in the presence of phospholipid.

In case of gel filtered enzyme (fig. 11) different phospholipid concentrations (0.025, 0.05, 0.1 and 0.2 mg/ml) were tested to recover the enzyme activity. In the absence of phospholipid, ATPase activity is 23.2 μmol Pi mg^{-1} protein h^{-1}. The optimum phospholipid concentration is found to be 0.05 mg/ml, at which enzyme activity is 26.7 μmol Pi mg^{-1} protein h^{-1}. Further increase in enzyme activity has not been found with the increase in phospholipid concentration.

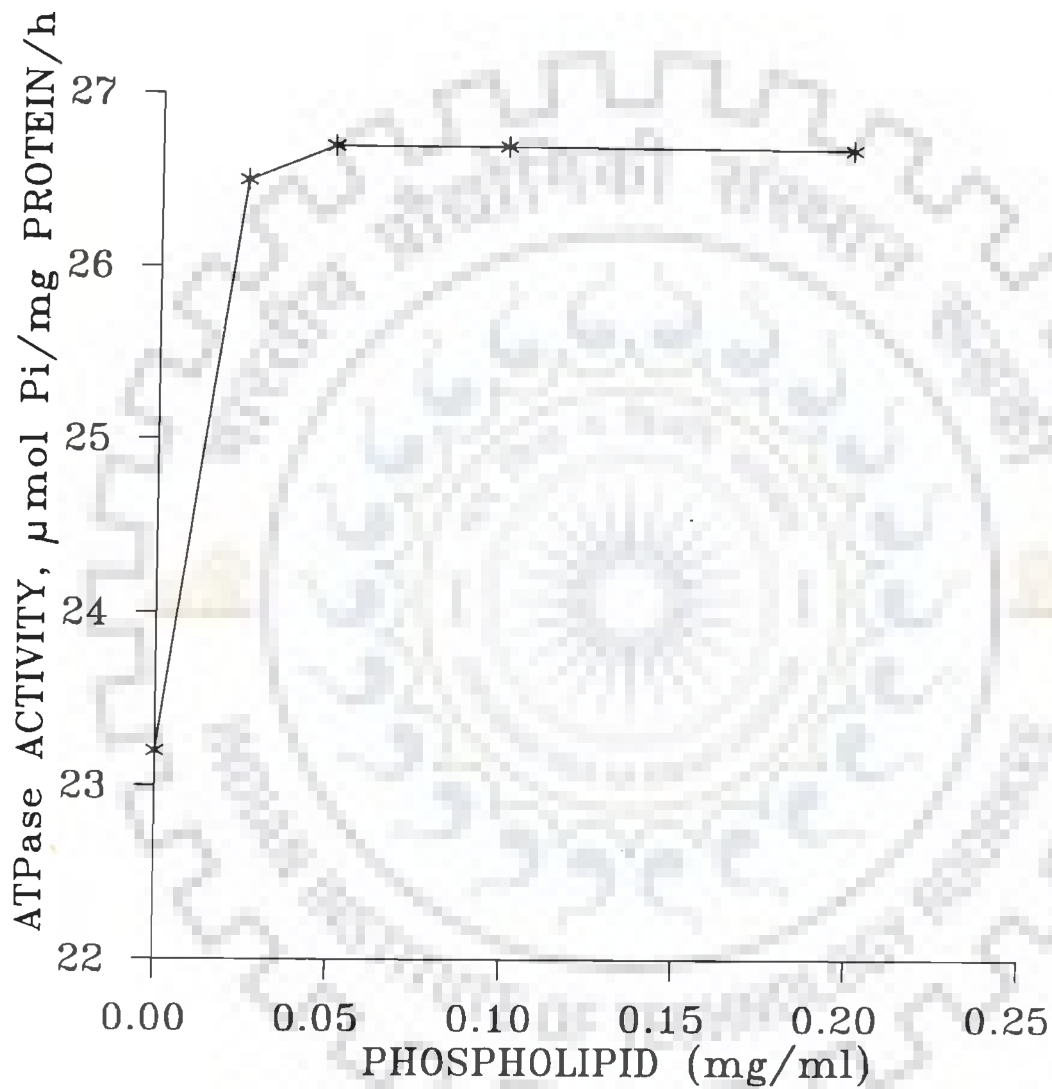


Fig. 11 Optimum phospholipid concentration for gel filtered enzyme.

4.9 Purification by gel filtration

1 ml of solubilized enzyme was loaded on Sepharose CL-6B column as described in previous Chapter. The void volume is 16 ml, which was measured by a completely excluded compound (Blue Dextran 2000). Fractions of 0.5 ml were collected and the protein content in them was determined by recording absorbance at 280 nm (Fig. 12). Absorbance starts increasing from fraction no. 35 and reaches the peak at fraction no. 58. Protein is completely eluted within 9 hours. ATPase activity is monitored by determining the rate of inorganic phosphate liberation as described in methods. Fractions were assayed in the absence of additional phospholipids. However the elution buffer contained 0.1 mg phospholipid/ml. Fractions 48-51 containing most of ATPase activity were combined and are referred as tonoplast ATPase peak. ATPase peak was assayed in presence of 0.05 mg/ml phospholipid.

The enzyme peak is obtained approximately in the middle of elution of thyroglobulin (669 kDa) and apoferretin (440 kDa) reference proteins which were run simultaneously on the column.

4.10 Degree of purification of the enzyme

In table 11, the data on degree of purification of the enzyme in comparison to the microsomal pellet are given. Tonoplast was separated from other membrane fractions like plasma membrane, Golgi apparatus and endoplasmic reticulum by 3-step sucrose gradient centrifugation as described in methods. This step resulted in separation of a tonoplast membrane enriched fraction at 15/ 35% sucrose interface (membrane fraction II). In this fraction, membrane bound ATPase is purified by 1.72 fold over the microsomal pellet and 27.4% of the total enzyme is recovered. In contrast the amount of protein content in membrane fraction II is less *i.e.* 16% of the microsomal protein. When membrane fraction II is solubilized with detergent (5% Triton X-

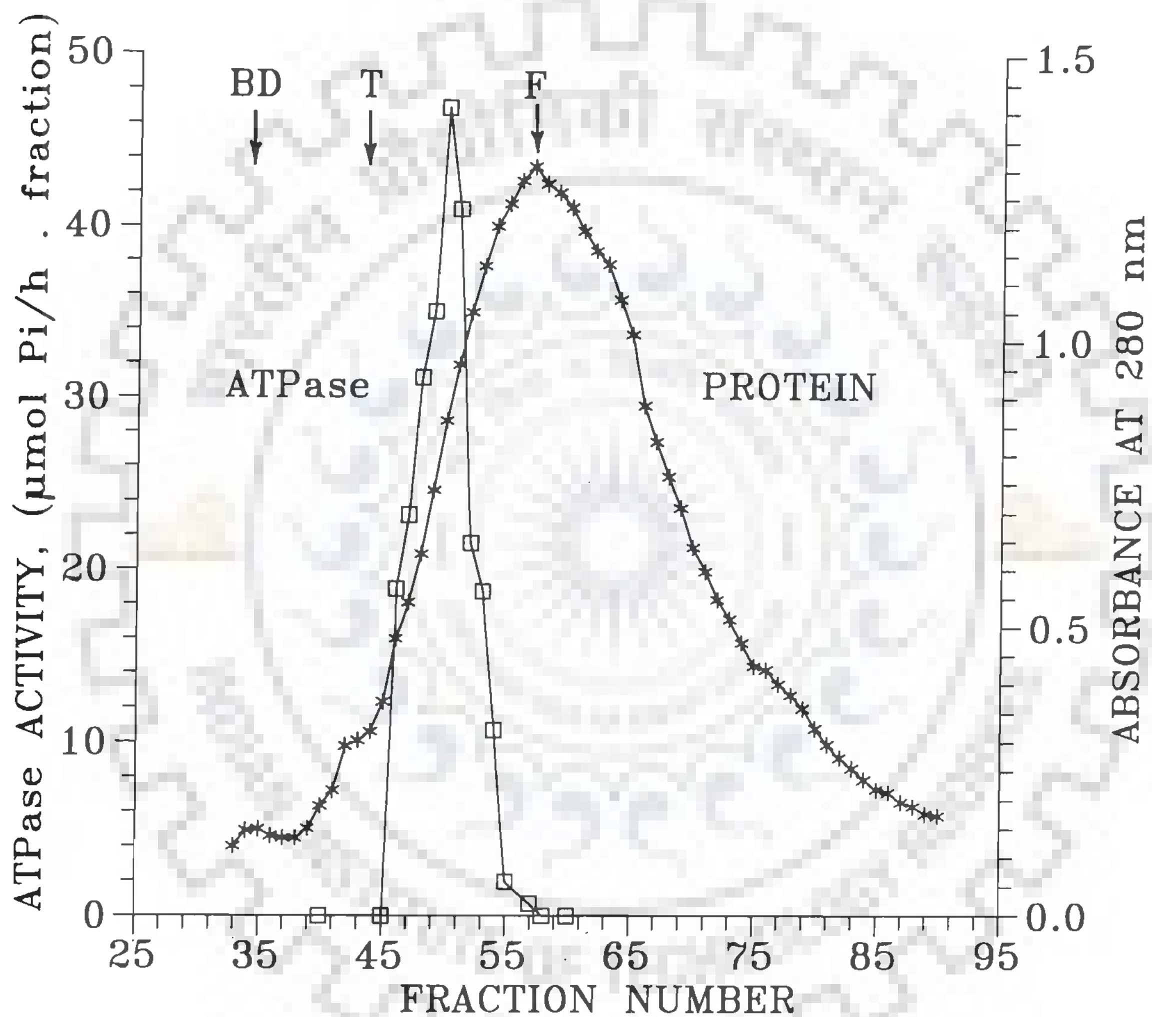


Fig. 12 **Gel filtration of Triton solubilized fraction II associated tonoplast ATPase activity on Sepharose CL-6B column.**

Arrows indicate position of blue dextran 2000 (BD), thyroglobulin (T) (molecular mass = 669 kDa), and apoferritin (F) (molecular mass = 440 kDa).

Table 11. Purification of tonoplast ATPase

Fraction	Total protein (mg) (%)	ATPase activity		Fold purification
		Total activity (μmol Pi h^{-1})(%)	Specific activity (μmol Pi mg^{-1} h^{-1})	
Microsomal pellet	10.33 (100)	26.46 (00)	2.56±0.56	1.00
15/35% sucrose interface	1.65 (16)	7.26 (27.4)	4.40±0.52	1.72
5% Triton X-100 extract	1.15 (11)	5.00 (19)	4.34±0.30	1.69
Sepharose CL-6B peak[1]	0.062 (0.6)	1.72 (6.5)	27.74±0.50	10.84
Calculated[2]	0.062 (0.6)	4.31 (16.3)	69.51±0.76	27.15

1 Fractions equivalent to 48-51 were combined and referred as peak

2 The calculated ATPase activities take into account the 60% loss in activity during gel filtration.

100), there is further loss of protein - only 11% of the microsomal protein is extracted in solubilized membrane fraction. 19% of the enzyme activity is recovered in 5% Triton X-100 extract. Solubilization step does not result in further purification of the enzyme. Solubilized tonoplast ATPase is then subjected to Sepharose CL-6B column, as described in methods. Gel filtration results in 10.84 fold purification of the tonoplast ATPase. Protein content of the peak tonoplast ATPase is only 0.6% of the protein of microsomal pellet and the enzyme activity yield is 6.5%. During the time taken for the completion of gel filtration step *i.e.* 8 hours, 60% of the enzyme activity is lost (fig. 13). Taking into account the 60% loss of enzyme activity, the total yield of the enzyme becomes 16.3% and the purification of the tonoplast ATPase becomes 27.15 fold.

4.11 Stability of the enzyme

Figure 14 shows that the step gradient purified enzyme is fairly stable and the activity is retained upto nearly six weeks at 4°C. At this temperature 5% and 25% of the activity is lost after 1st and 2nd day respectively. However, thereafter, activity remains almost stable upto 7th day. A steady and uniform reduction of activity is noted from 7th day onward. About half of the activity is retained even on 22nd day. On 45th day 11% activity still remains. At -20°C, no marked change in enzyme activity is observed for first two days. Significant amount of loss of enzyme activity (32%) is noted on 3rd day. About 10% decrease in activity is seen between 3rd to 6th day. The rate of change of enzyme activity is relatively much lower between 6th and 29th day. However, similar observations can be made even beyond 29th day, though the rate of loss of activity is slightly higher. It may be mentioned here that at -20°C, the enzyme activities on 29th and 45th day are 13.62 and 7.68 μmol Pi mg^{-1} protein h^{-1} respectively which are significantly higher compared to the enzyme activities at 4°C. However the enzyme is much less

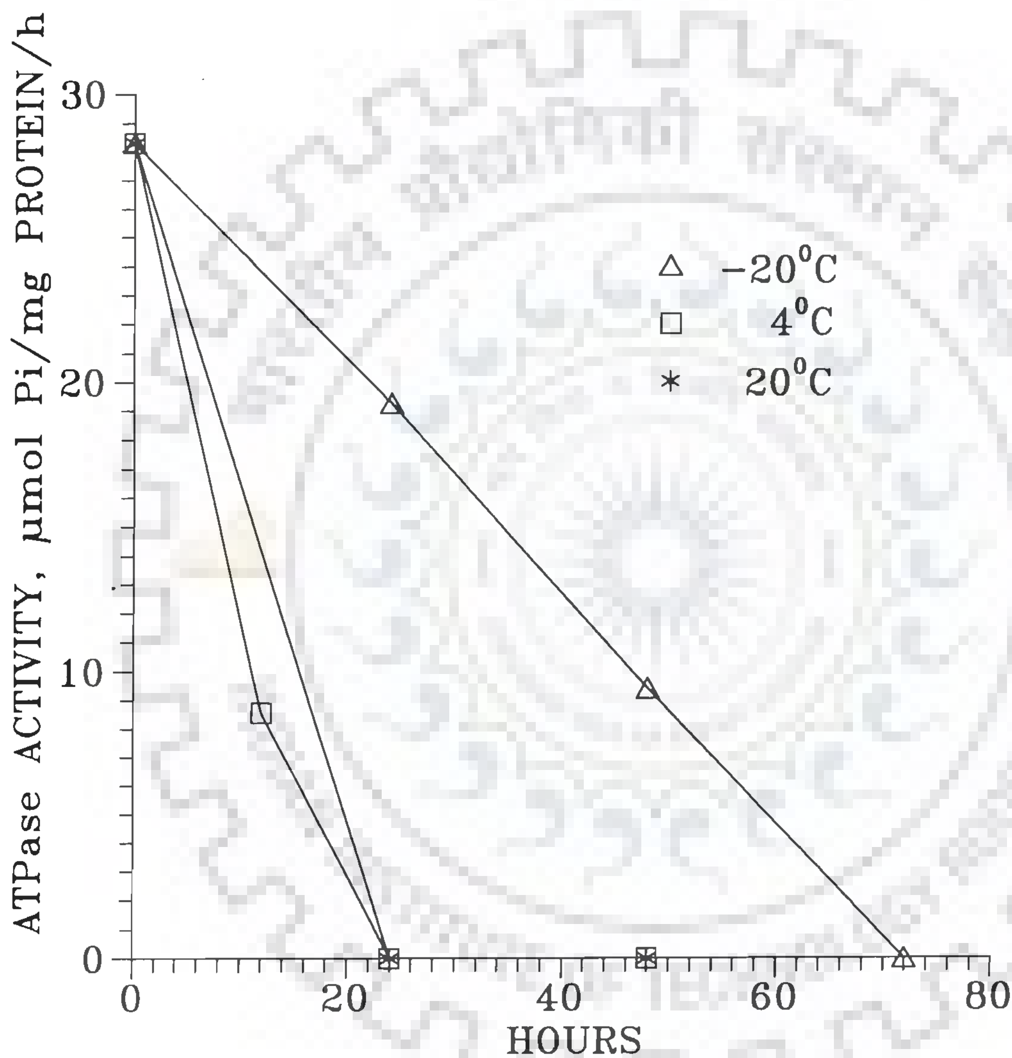


Fig. 13 Stability of tonoplast ATPase after purification through Sepharose CL-6B column.

Stability was checked at three different temperatures -20°C, 4°C and 20°C. Symbols for different temperatures are shown in the figure.

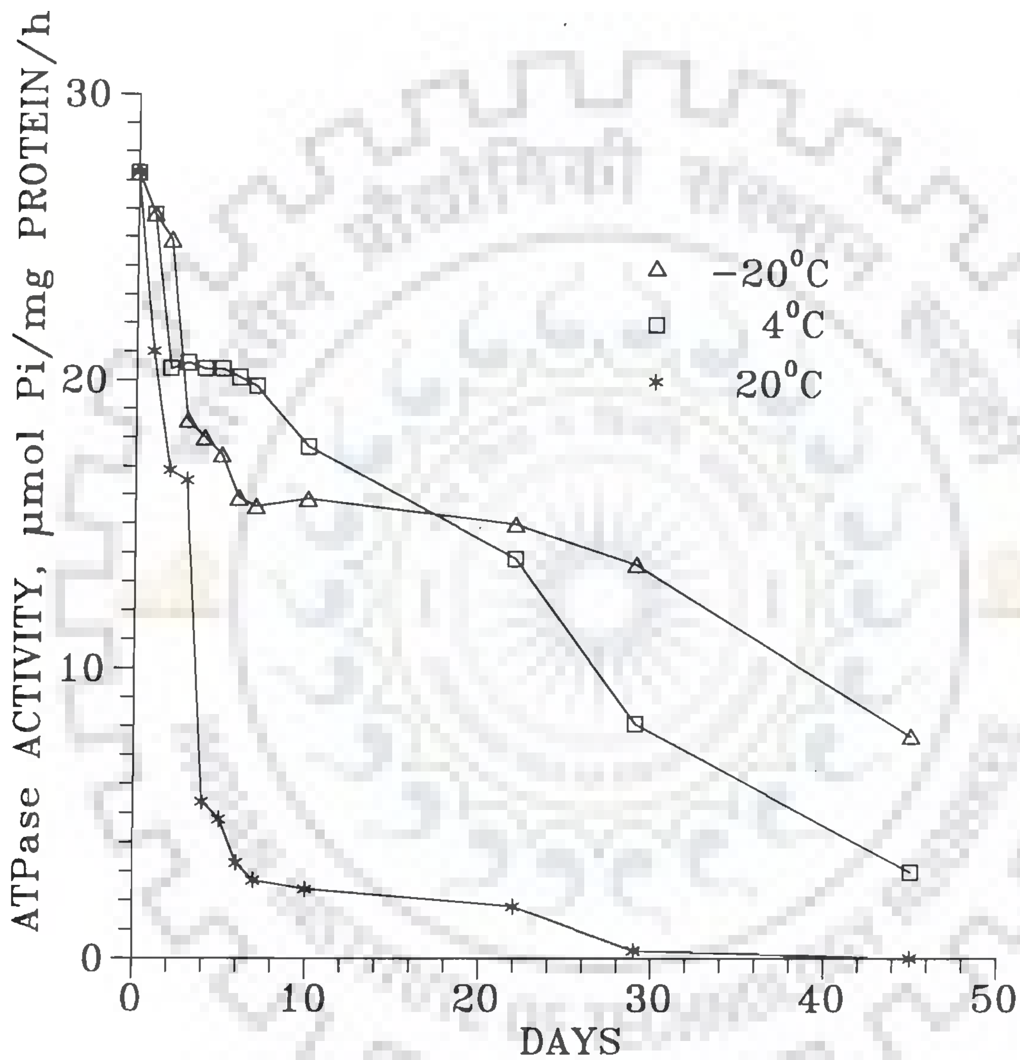


Fig. 14 Stability of tonoplast ATPase after step-gradient purification.

Stability was checked at three different temperatures -20°C, 4°C and 20°C. Symbols for different temperatures are shown in the figure.

stable at 20°C. At this temperature, the enzyme activity is reduced from 27.27 to 16.5 μmol Pi mg^{-1} protein h^{-1} (i.e. 60% reduction in enzyme activity) after three days and the same goes down drastically to 5.4 μmol Pi mg^{-1} protein h^{-1} causing 20% reduction in enzyme activity on 4th day. On 5th day onward, ATPase activity gradually decreases and becomes almost negligible after four weeks.

By contrast the gel filtered enzyme (fig. 13) is highly unstable and looses all its activity within 24 h at both 20°C and 4°C. At -20°C, 32% enzyme activity is lost after 24 h. After 48 h, 33% enzyme activity still remains and activity totally diminishes after 72 h.

4.12 Homogeneity and determination of molecular weight by SDS-PAGE

The homogeneity of the enzyme fractions obtained at different steps of purification was tested by SDS-PAGE (fig. 15). Tonoplast membrane fraction, obtained from sucrose density gradient, and solubilized membrane fraction were loaded in lane 1 and 2 respectively. Lane 8 contains molecular weight marker. Lane 3-7 shows the pattern of bands of different fractions eluted from Sepharose CL-6B column. Fractions 44-45 do not show any ATPase activity and also it is found that Lane 3 does not show 69 and 55 kDa bands. Fractions 48-49, 50-51 constitute the peak of tonoplast ATPase amongst which fractions 50-51 show highest activity. 69 and 55 kDa bands are present in both Lane 4 and 5, constituting the fractions 48-49 and 50-51 respectively. But in Lane 5, fractions 50-51, having maximum tonoplast ATPase activity shows only 3 major bands - 69, 55 and 20 kDa, along with two minor bands 37 and 15 kDa; while other bands disappear (fig. 15). Band with apparent mass of 69 kDa, gradually shows less intensity in Lane 6 and 7 analysing the fractions 52-53 and 54-55 respectively. Thus from the pattern of various bands obtained by electrophoresis, it appears that purified tonoplast ATPase consists of at least three polypeptides of 69, 55 and 20 kDa. Copurification of minor components

Fig. 15 SDS-PAGE photograph of tonoplast ATPase of various fractions collected from Sepharose CL-6B column.

TP and TPs denote tonoplast membrane purified on 3 step sucrose gradient i.e. fraction II and Triton solubilized fraction II respectively. ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}.

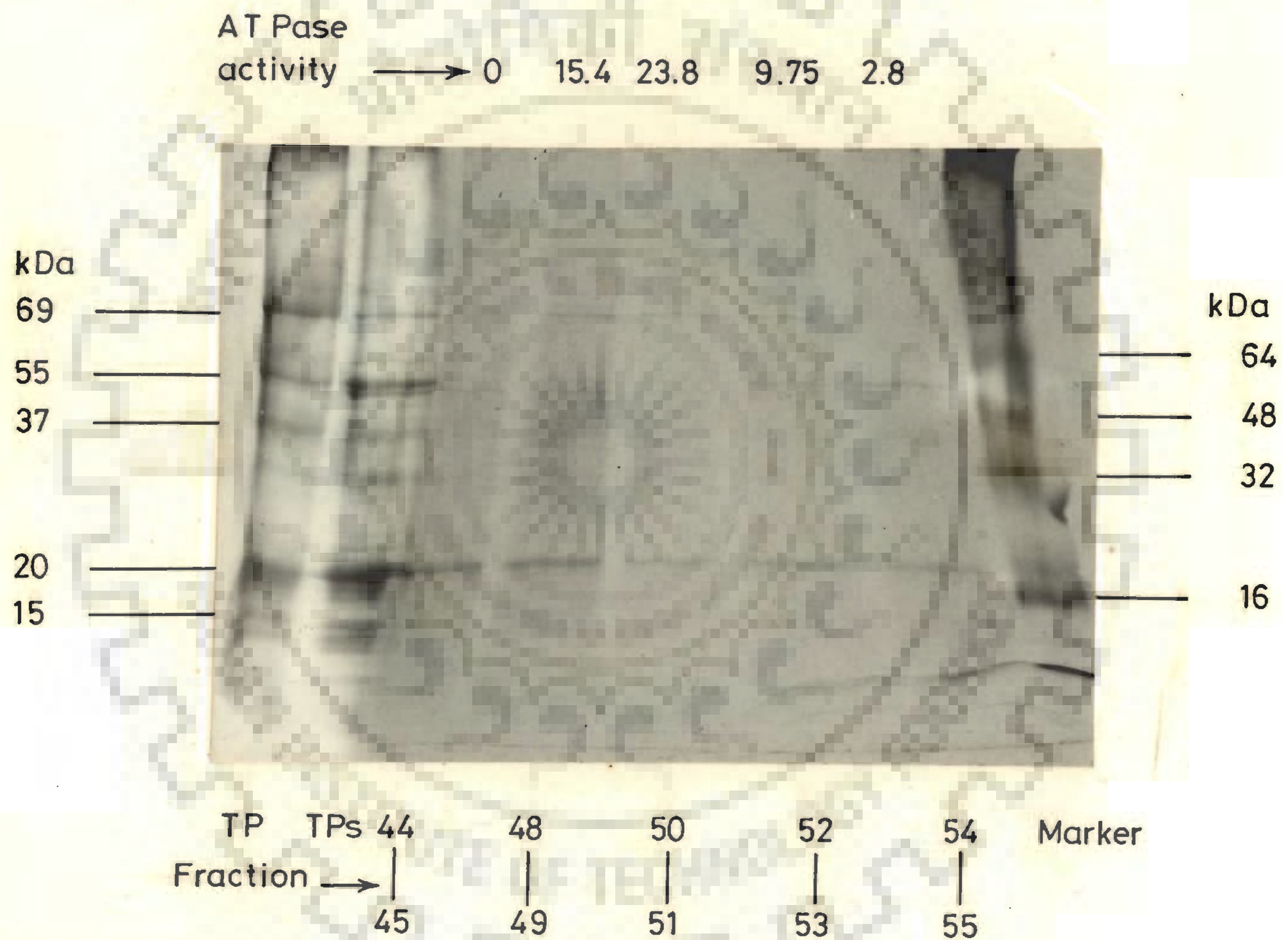


FIG. 15

like 37 and 15 kDa along with the other three major polypeptides implies that peanut tonoplast ATPase is multimeric enzyme which is made up of as many as five polypeptides.

4.13 Interaction with plant hormones

To check the effect of various plant hormones on tonoplast ATPase activity, two approaches were undertaken. Firstly the tonoplast enriched membrane fraction was isolated, various hormones were added to this fraction, these samples were incubated for specific time periods and the enzyme activity was measured. The second approach was to treat the seeds directly with different hormone solutions and the treated seeds were germinated. The details of these methods are described in section **3.2.10**.

Fig. 16a-c show the growth pattern of the seedlings germinated from the seeds treated with different hormone solutions. Growth of the seedlings is mostly retarded in presence of kinetin. Seeds treated with IBA, NAA and ABA also show lesser growth than the control. Much differentiated growth pattern has not been observed in case of IAA, PAA and GA_3. Application of different concentrations of hormones does not cause any significant variation in size of the growing seedlings.

Effects of addition of several plant hormones on membrane preparation enriched in tonoplast ATPase are shown in fig. 17 and table 12. In presence of 10^{-4} and 10^{-5} M concentrations of IAA, ATPase activity is 5.83 and 5.62 μmol Pi mg^{-1} protein h^{-1} respectively which is 81 and 78% of the activity compared to control without hormones. With increase in IAA concentration (10^{-3} M) the inhibition is more *i.e.* 45% inhibition of enzyme activity. Of the various hormones, application of only PAA is stimulatory to tonoplast ATPase activity, for example PAA at various concentrations *i.e.* 10^{-3}, 10^{-4} and 10^{-5} M enhances the tonoplast ATPase activity by 1.7-1.8 fold. Other analogues of IAA *i.e.* IBA and NAA are also act as inhibitors

Fig. 16 **Growth pattern of the seedlings, treated with (a) 10^{-3} M, (b) 10^{-4} M and (c) 10^{-5} M hormone solutions.**

FIG. 16

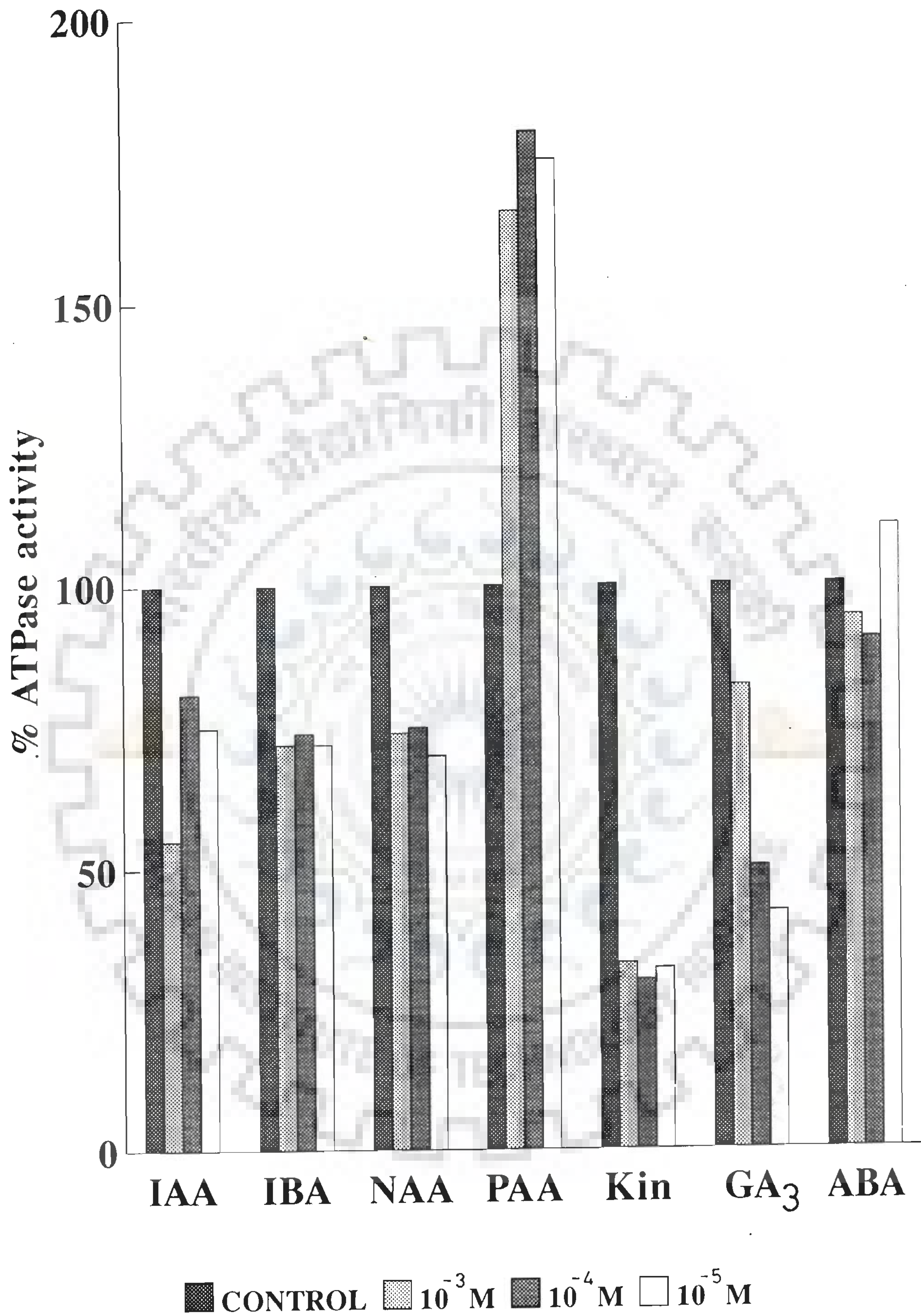


Fig. 17 **Effect of plant hormones, *in vitro* (details given in methods), on tonoplast ATPase.**

Three different concentrations (10^{-3}, 10^{-4} and 10^{-5} M) were used for each hormone. Different hormones were added in assay medium.

Table 12. Effect of Plant hormones, *in vitro*, on tonoplast ATPase

Hormone	ATPase activity* at different concentrations of hormones		
	10^{-3}M	10^{-4}M	10^{-5}M
IAA[1]	3.96	5.83	5.62
IBA[1]	5.18	5.33	5.18
NAA[1]	5.33	5.40	5.04
PAA[1]	11.95	12.96	12.60
Kinetin[1]	2.38	2.16	2.30
GA_3[2]	5.74	3.50	2.94
ABA[1]	6.77	6.48	7.92

* ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}

1 Activity in control (50 mM Tris-MES [pH 8.0], 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl, 100 μM ammonium molybdate, 0.01 N NaOH) is 7.2 μmol Pi mg^{-1} protein h^{-1}.

2 Activity in control (50 mM Tris-MES [pH 8.0], 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl, 100 μM ammonium molybdate, 0.5% ethylalcohol) is 7.0 μmol Pi mg^{-1} protein h^{-1}.

of enzyme activity at various concentrations. Other hormones *i.e.* kinetin and GA_3 as well inhibit tonoplast ATPase activity. Inhibitory effect of kinetin at various concentrations is quite consistent, for example application of 10^{-3}, 10^{-4} and 10^{-5} M kinetin results in 67, 70 and 68% inhibition of enzyme activity. GA_3 is less inhibitory at 10^{-3} M. At 10^{-3} M GA_3, 82% of the enzyme is active. However at lower concentrations *i.e.* 10^{-4} and 10^{-5} M GA_3, only 50 and 58% inhibition is observed respectively. ATPase enzyme shows either little (6-10%) or no inhibition when treated with ABA at 10^{-3}, 10^{-4} and 10^{-5} M concentrations.

Fig. 18 and table 13 depict the effects of various plant hormones on tonoplast ATPase when the seeds are treated with different hormones. The results reveal that IAA at 10^{-4} and 10^{-5} concentrations causes 55% inhibition of enzyme activity, the inhibition is more pronounced *i.e.* 75% at higher concentration *i.e.* 10^{-3} M. Both IBA and NAA at various concentrations are more inhibitory to the enzyme. AT all the three concentrations these hormones cause 65-72% reduction of enzyme activity. PAA is least inhibitory and causes only 9-14% inhibition of enzyme at various concentrations. A comparison of these data with the experiment in which various auxins were added to membrane preparation reveals that the hormonal treatment if given directly to seeds results in much more pronounced inhibition of tonoplast ATPase activity. In fact PAA which acts as stimulatory if added to membrane preparation causes slight inhibition of enzyme activity if applied to seeds. Treatment with kinetin results in 80% or more loss of enzyme activity. ABA also causes about 50% inhibition in enzyme activity. Thus like auxins the inhibitory effect of both kinetin and ABA become much more pronounced if these hormones are applied to seeds. By contrast GA_3 increases ATPase activity marginally. On the other hand, as noted earlier this hormone is inhibitory if added to membrane preparations.

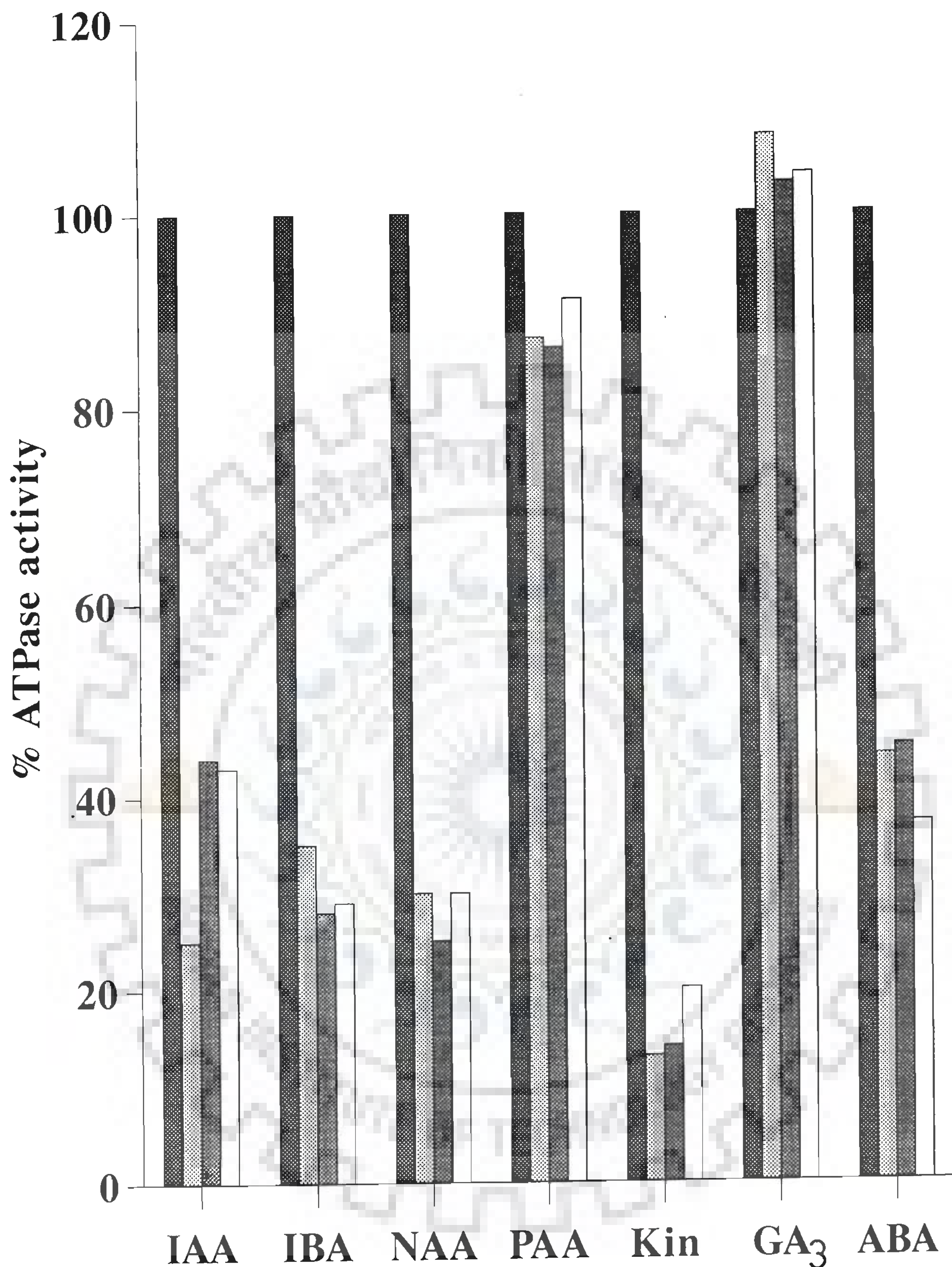


Fig. 18 Effect of plant hormones, *in vivo* (details given in methods), on tonoplast ATPase.

Three different concentrations (10^{-3}, 10^{-4} and 10^{-5} M) were used for each hormone. Seeds were incubated for 24 h in hormone solutions and then germinated in plant growth chamber for definite period.

Table 13. Effect of Plant hormones, *in vivo*, on tonoplast ATPase

Hormone	ATPase activity* at different concentrations of hormones		
	10^{-3}M	10^{-4}M	10^{-5}M
IAA[1]	1.73	3.04	2.97
IBA[1]	2.42	1.93	2.00
NAA[1]	2.07	1.73	2.07
PAA[1]	6.00	5.93	6.28
Kinetin[1]	0.90	0.97	1.38
GA_3[2]	7.45	7.45	7.59
ABA[1]	3.04	3.10	3.24

* ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}

1 Activity in control is 6.9 μmol Pi mg^{-1} protein h^{-1}.

2 Activity in control is 6.85 μmol Pi mg^{-1} protein h^{-1}.

Assay buffer - 50 mM Tris-MES [pH 8.0], 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl, 100 μM ammonium molybdate.

4.14 Interaction with phenolic compounds

Effect of addition of phenolic compounds to membrane preparations containing tonoplast ATPase is shown in fig. 19 and table 14. Chlorogenic acid at all three concentrations (10^{-3}, 10^{-4} and 10^{-5} M) completely inhibits the tonoplast ATPase activity. At 10^{-3} M concentration both hydroquinone and hydroxybenzoic acid as well show complete inhibition. At 10^{-4} and 10^{-5} M concentrations of hydroxybenzoic acid, the enzyme activity is reduced from 7.2 (activity in control) to 5.83 and 5.4 μmol Pi mg^{-1} protein h^{-1} respectively *i.e.* about 20% inhibition of enzyme activity is resulted. In case of hydroquinone inhibition of ATPase activity is notably decreased with the decrease in its concentration. At 10^{-4} and 10^{-5} M concentrations of hydroquinone, enzyme activity is 3.07 and 8.28 μmol Pi mg^{-1} protein h^{-1} respectively *i.e.* enzyme activity is reduced by 66 and 8% respectively. A marked increase in enzyme activity is observed when cinnamic acid is applied. The increase in enzyme activity is about 1.2 fold when concentration of cinnamic acid is 10^{-3} M and the same is about 1.8 fold at lower concentrations (10^{-4} and 10^{-5} M).

Seeds are unable to germinate when incubated with phenolic compounds (hydroquinone, hydroxybenzoic acid, cinnamic acid and chlorogenic acid) at any of the three different concentrations *i.e.* 10^{-3}, 10^{-4} and 10^{-5} M (fig. 20a-c). Only seedlings treated with 10^{-4} and 10^{-5} M concentrations of hydroquinone show little growth (fig. 20b,c). Determination of tonoplast ATPase activity in the seedlings show that the enzyme is about 10% active while compared to the control (fig. 21 and table 15).

4.15 Ultrastructure of tonoplast ATPase under transmission electron microscope

Ultrastructure of the tonoplast ATPase is observed under transmission electron microscope by the help of negative staining. When negative stain is applied to the grid, the stain

Table 14. **Effect of phenolic compounds, *in vitro*, on tonoplast ATPase**

Phenolic coumpounds	ATPase activity* at different concentrations of phenolic compounds		
	10^{-3}M	10^{-4}M	10^{-5}M
Hydroquinone[1]	0	3.07	8.28
Hydroxybenzoic acid[2]	0	5.83	5.40
Cinnamic acid[3]	8.61	12.78	12.89
Chlorogenic acid[1]	0	0	0

* ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}

1 Activity in control (50 mM Tris-MES [pH 8.0], 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl, 100 μM ammonium molybdate) is 7.2 μmol Pi mg^{-1} protein h^{-1}

2 Activity in control (50 mM Tris-MES [pH 8.0], 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl, 100 μM ammonium molybdate, 0.01 N NaOH) is 7.2 μmol Pi mg^{-1} protein h^{-1}

3 Activity in control (50 mM Tris-MES [pH 8.0], 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl, 100 μM ammonium molybdate, 0.5% methanol, 0.01% Triton X-100) is 7.3 μmol Pi mg^{-1} protein h^{-1}

Fig. 19 Effect of phenolic compounds, *in vitro* (details given in methods), on tonoplast ATPase.

Three different concentrations (10^{-3}, 10^{-4} and 10^{-5} M) were used for each phenolic compound. Different phenolic compounds were added in assay medium.

HQ - Hydroquinone
HB - Hydroxybenzoic acid
CIN - Cinnamic acid
CHL - Chlorogenic acid

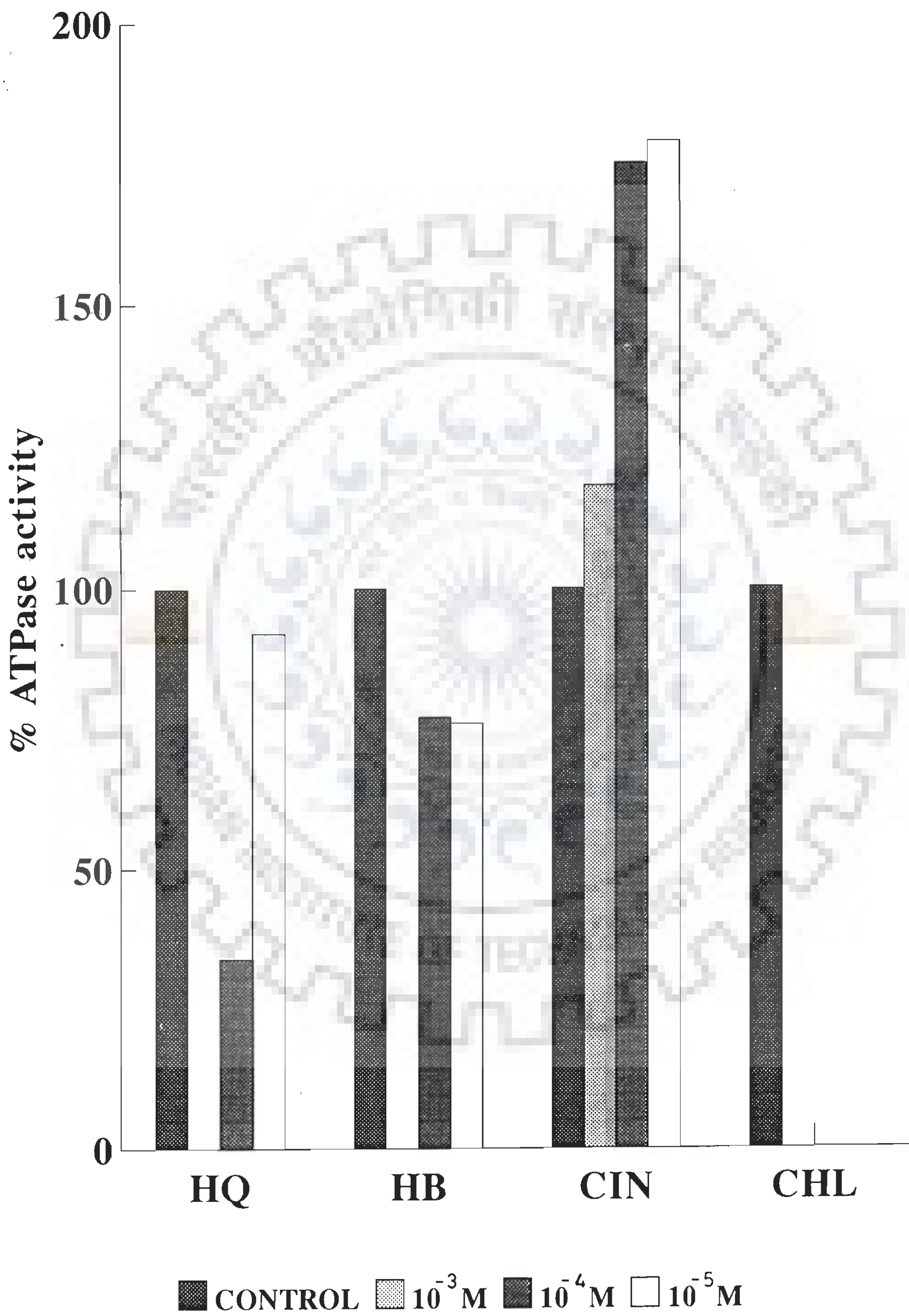


Fig. 19

Fig. 20 **Growth pattern of the seedlings, treated with (a) 10^{-3} M, (b) 10^{-4} M and (c) 10^{-5} M solutions of phenolic compounds.**

Control
Hydroquinone
Hydroxybenzoic acid
Cinnamic acid
Chlorogenic acid
a

Control
Hydroquinone
Hydroxybenzoic acid
Cinnamic acid
Chlorogenic acid
b

Control
Hydroquinope
Hydroxybenzoic acid
Cinnamic acid
Chlorogenic acid
c

FIG. 20

Table 15. **Effect of phenolic compounds, *in vivo*, on tonoplast ATPase**

Phenolic coumpounds	ATPase activity* at different concentrations of phenolic compounds		
	10^{-3}M	10^{-4}M	10^{-5}M
Hydroquinone[1]	0	0.65	0.72
Hdroxybenzoic acid[2]	0	0	0
Cinnamic acid[3]	0	0	0
Chlorogenic acid[1]	0	0	0

* ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}

1 Activity in control is 6.5 μmol Pi mg^{-1} protein h^{-1}

2 Activity in control is 6.55 μmol Pi mg^{-1} protein h^{-1}

3 Activity in control is 4.1 μmol Pi mg^{-1} protein h^{-1}

Assay buffer - 50 mM Tris-MES [pH 8.0], 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl, 100 μM ammonium molybdate.

Fig. 21 **Effect of phenolic compounds, *in vivo* (details given in methods), with tonoplast ATPase.**

Three different concentrations (10^{-3}, 10^{-4} and 10^{-5} M) were used for each phenolic compound. Seeds were incubated for 24 h in phenolic solutions and then germinated in plant growth chamber for definite period.

HQ - Hydroquinone
HB - Hydroxybenzoic acid
CIN - Cinnamic acid
CHL - Chlorogenic acid

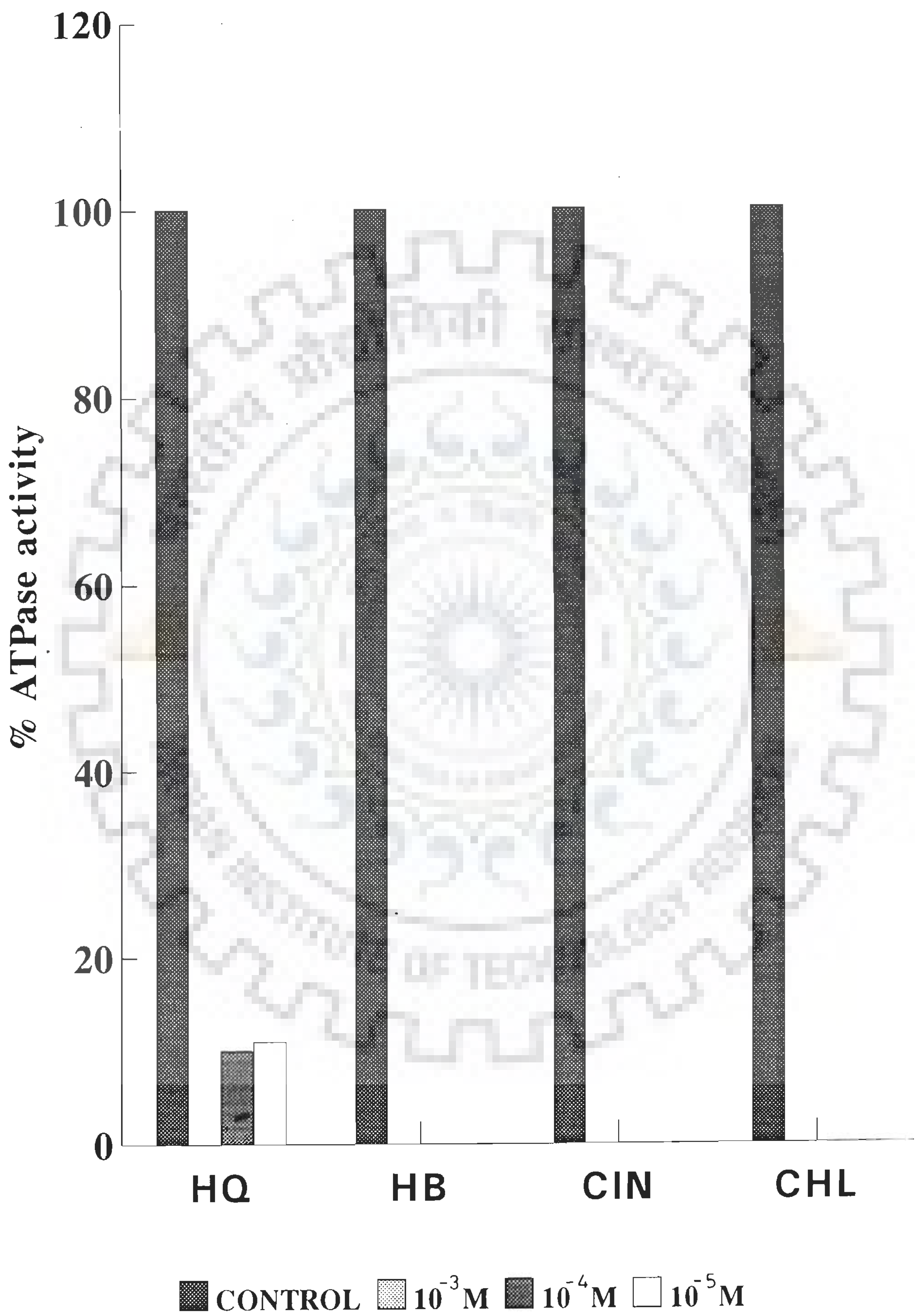


Fig. 21

coats the specimen, reproducing its topography and filling in the background. As the solvent evaporates, it leaves behind a smooth film of heavy metal. As the specimen is held within this dense supporting matrix, it gets preserved. It may be mentioned here that specimen is always mounted on a coated grid. Application of negative stain on carbon coated grid ensures that sufficient density is imparted to the specimen for interaction with the electron beam. In this study, 1% PTA was used as negative stain.

Purity of the membrane preparation was checked before examining the ultrastructure of the membrane bound enzyme under transmission electron microscope. Tables 16 and 17 show the inhibitor sensitivity characteristics of the tonoplast ATPase and the mitochondrial ATPase respectively. Tonoplast enriched fraction is inhibited (49%) by nitrate. Inhibitors of mitochondrial and plasma membrane ATPase *i.e.* azide and vanadate respectively do not cause any significant inhibition (<10%) of tonoplast ATPase. Similarly membrane preparation used for examining mitochondrial ATPase is markedly inhibited (74%) by azide, whereas nitrate and vanadate cause less than 5% inhibition. Thus vacuolar and mitochondrial membrane preparations are not significantly contaminated by other membrane fractions.

When negatively stained membrane fraction is observed under electron microscope, numerous flattened vesicles, irregular in outline are found associated with dense particles over their surfaces. Higher magnification (45,000X) reveals the head and stalk structures of the dense particles (fig. 22a). These stalked structures are scattered discretely on the surface as well as on the edge of the vesicle (fig. 22b). Particles vary in size, some shorter structures are also present. The enzyme particles seem to vary in shape too. Although majority of them exhibit bilateral symmetry with the cleft down the middle, some appear as a pair of a shorter structure emerging from the stalk (fig. 22a) and very few are pyramidal in shape (fig. 22a,b). The most important

Table 16. **Effect of different inhibitors on membrane fraction II bound ATPase used for observation of ultrastructure of tonoplast ATPase under transmission electron microscope**

Inhibitor	ATPase activity*	% ATPase activity	% inhibition
Control[1]	11.67	100.00	-
Nitrate (50 mM)	5.96	51.10	48.90
Azide (0.5 mM)	10.57	90.57	9.43
Vanadate (0.2 mM)	11.20	96.00	4.00

* ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}

1 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate

Table 17. **Effect of different inhibitors on mitochondrial membrane preparation used for observation of ultrastructure of the associated ATPase under transmission electron microscope**

Inhibitor	ATPase activity*	% ATPase activity	% inhibition
Control[1]	7.80	100.00	-
Nitrate (50 mM)	7.65	98.10	1.90
Azide (0.5 mM)	2.02	25.90	74.10
Vanadate (0.2 mM)	7.45	95.60	4.40

* ATPase activity is expressed as μmol Pi mg^{-1} protein h^{-1}

1 50 mM Tris-MES buffer (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate

feature is that there is distinct cleft or notch in the head portion of the tonoplast bound enzyme. In contrast, mitochondrial ATPase lacks cleft and is mostly spherical (fig. 22c). The diameter of the head piece of the tonoplast ATPase is 9.6 - 11.0 nm and length of the stalk is 8.0 - 8.5 nm, whereas the mitochondrial ATPase is slightly smaller with the head piece of 8.0 - 8.5 nm in diameter and the stalk of 6.0 nm in length. When the tonoplast membrane fraction is incubated for a brief period (20-30 min) in the Mg-ATP solution (Tris-MES, pH 8.0, ATP, $MgSO_4$, KCl, ammonium molybdate) and KNO_3 (end concentration, 100 mM), the enzyme tends to disappear from the vesicles (fig. 22d). Membrane fraction treated with azide (fig. 22e) and vanadate (fig. 22f) does not show loss of enzyme structure. In contrast, the mitochondrial ATPase when treated with nitrate does not show any visible effect (fig. 22g).

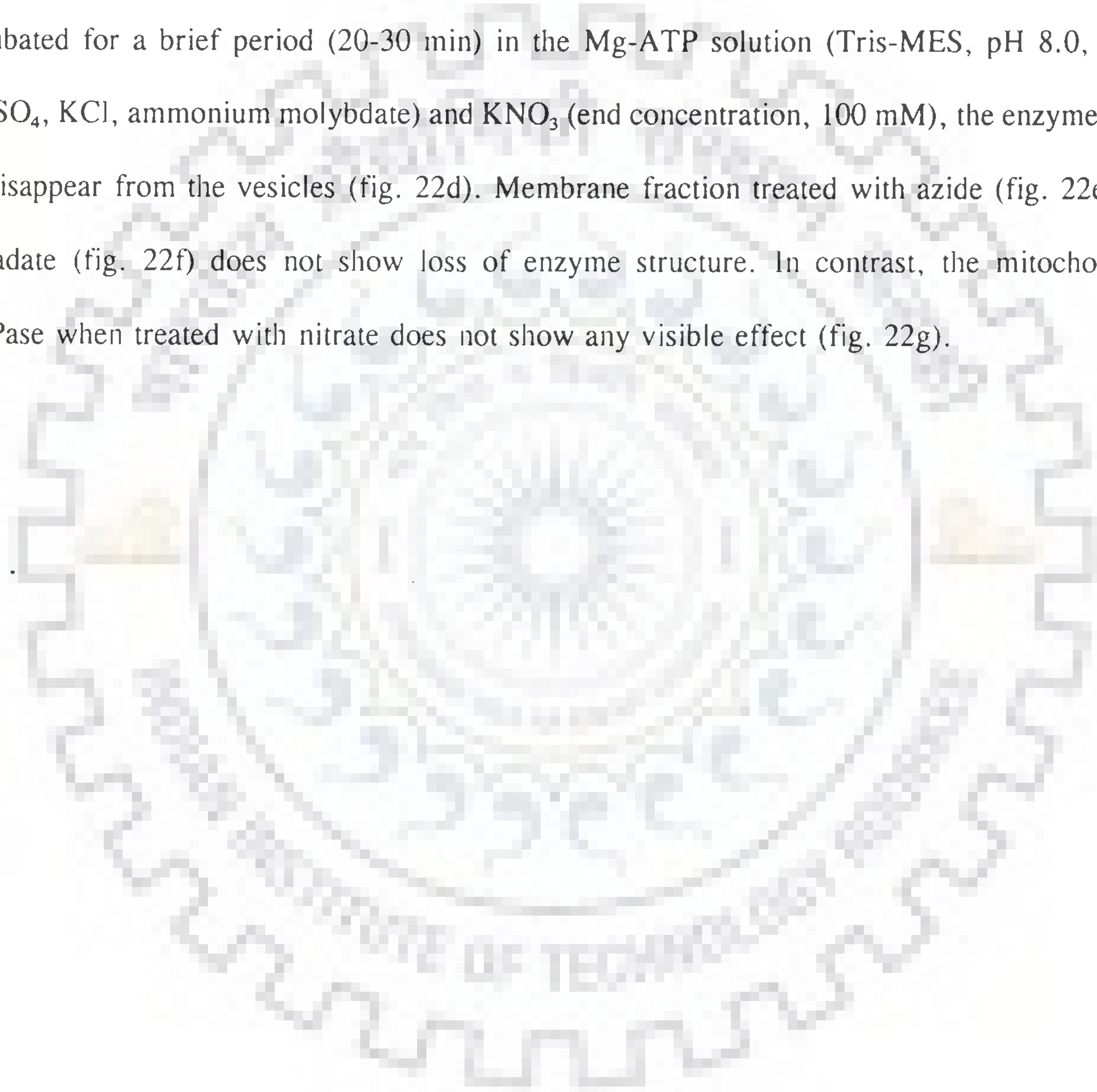
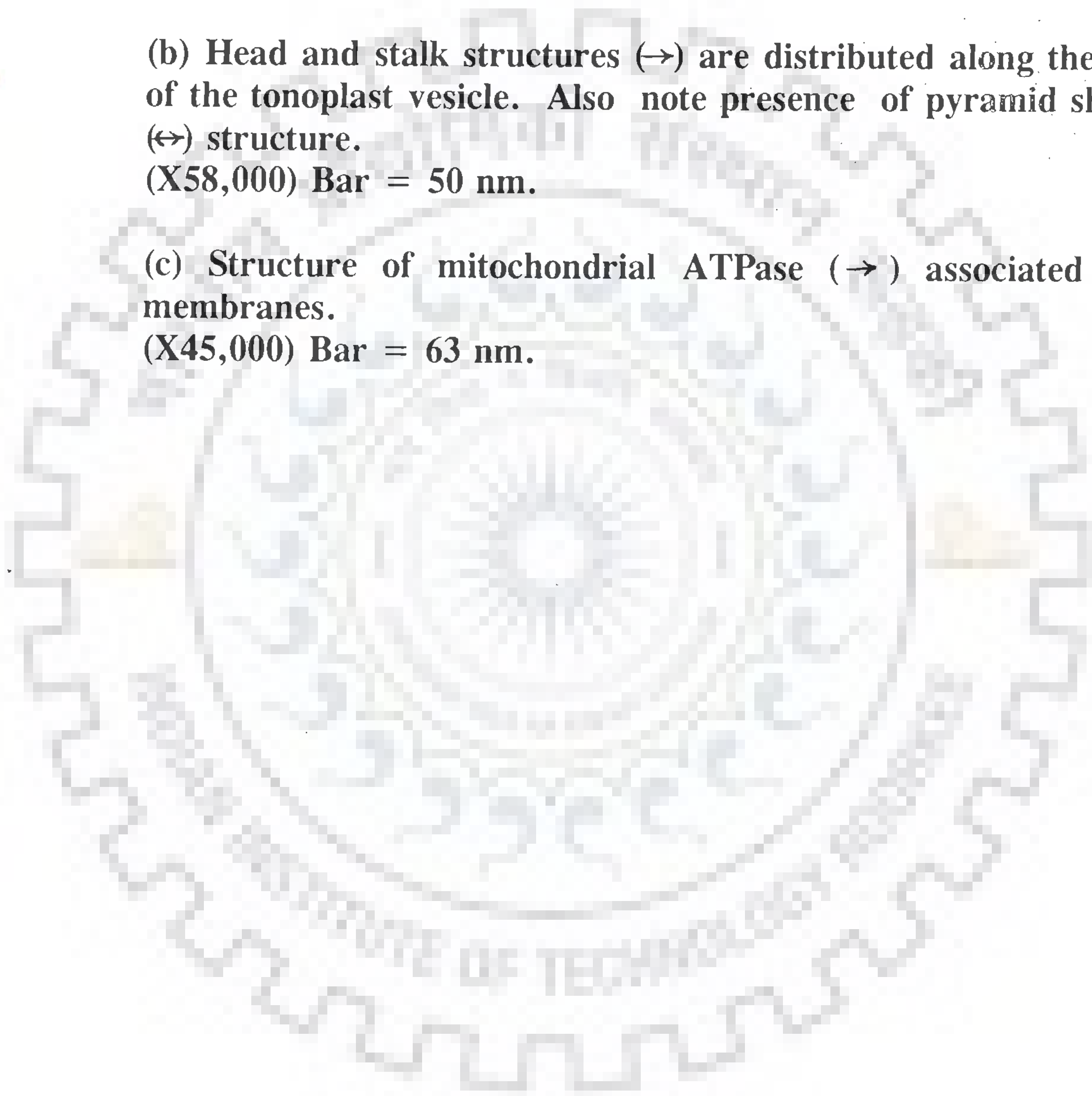


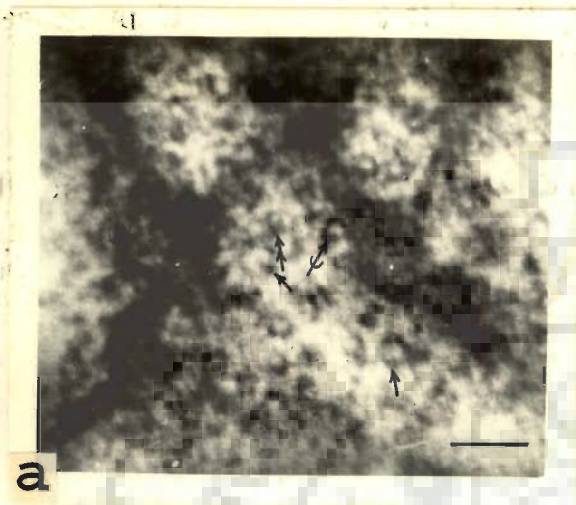
Fig. 22 **Negative-staining electron microscopy of the tonoplast enriched fraction and mitochondrial membrane preparation.**

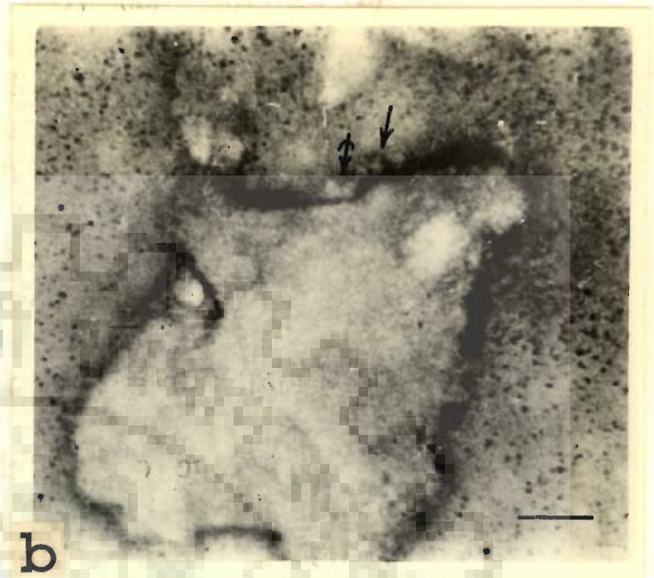
(a) The tonoplast vesicles are characterized by numerous stalked particles irregularly distributed along the outer membrane surface. The enzyme displays distinctive cleft (→). Also note presence of paired (⇉) and pyramid shaped (↔) structures.
(X58,000) Bar = 50 nm.

(b) Head and stalk structures (→) are distributed along the edge of the tonoplast vesicle. Also note presence of pyramid shaped (↔) structure.
(X58,000) Bar = 50 nm.

(c) Structure of mitochondrial ATPase (→) associated with membranes.
(X45,000) Bar = 63 nm.







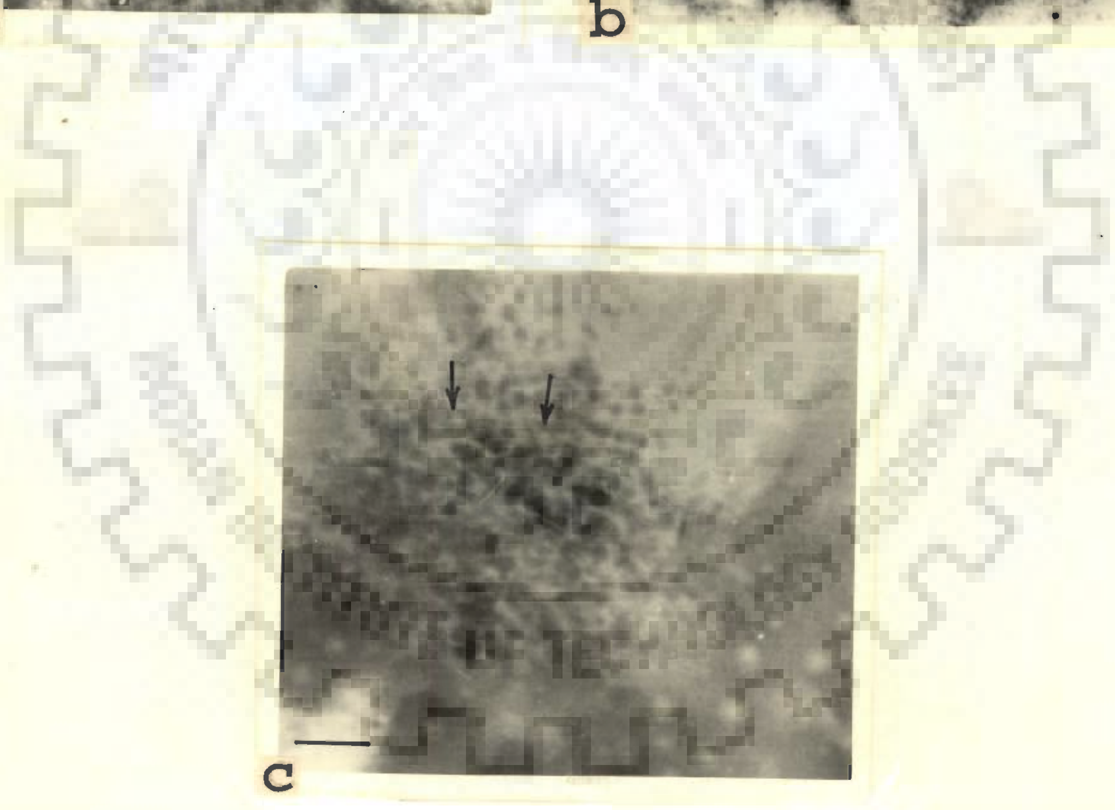


FIG. 22 a-c

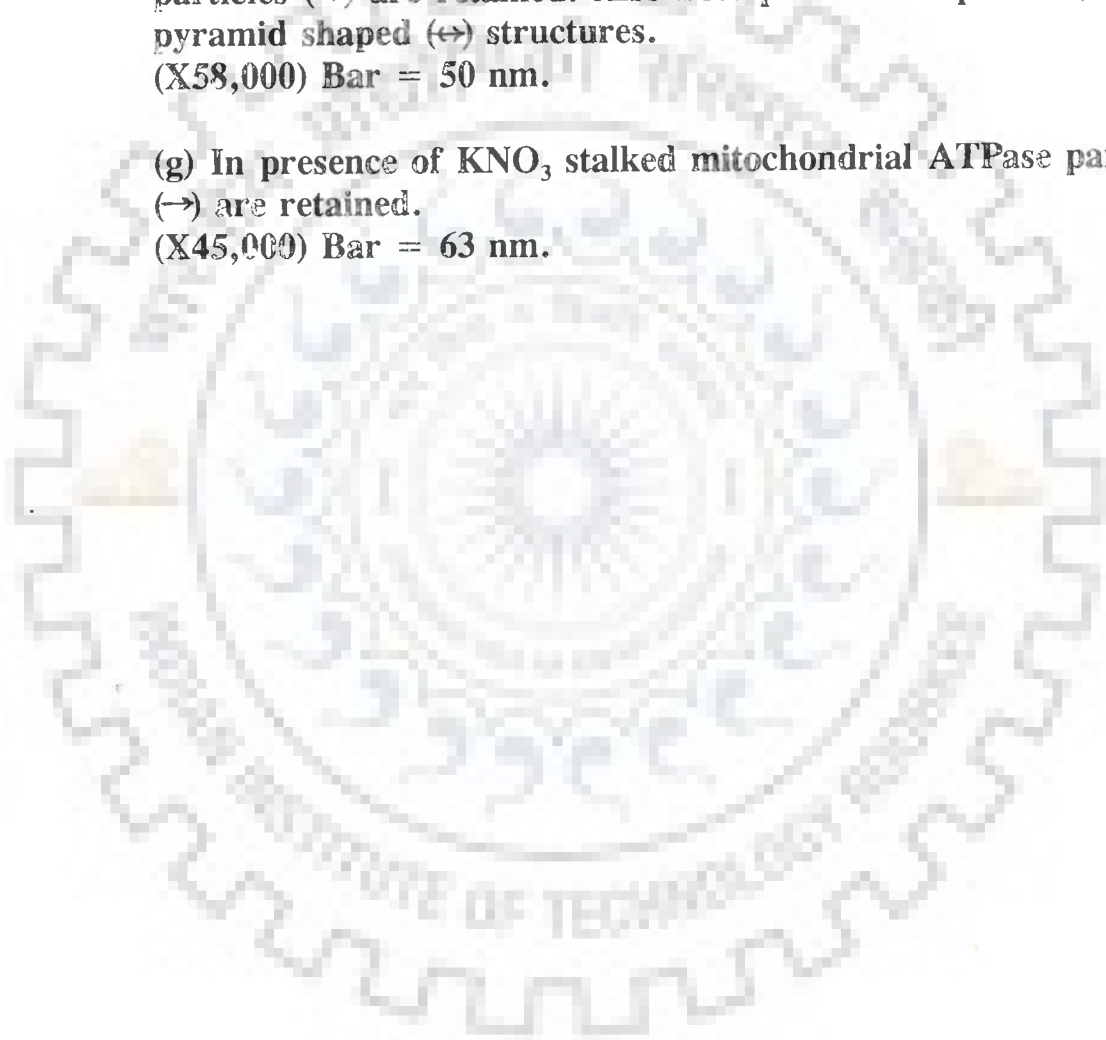
Fig. 22 (contd.)

(d) In presence of 100 mM KNO_3 head and stalk structure of tonoplast ATPase disappears from the surface of the vesicles (→). (X45,000) Bar = 63 nm.

(e) In presence of azide (0.5 mM), the stalked tonoplast ATPase particles (→) are retained.
(X58,000) Bar = 50 nm.

(f) In presence of vanadate (0.2 mM), stalked tonoplast ATPase particles (→) are retained. Also note presence of paired (↠) and pyramid shaped (↔) structures.
(X58,000) Bar = 50 nm.

(g) In presence of KNO_3 stalked mitochondrial ATPase particles (→) are retained.
(X45,000) Bar = 63 nm.



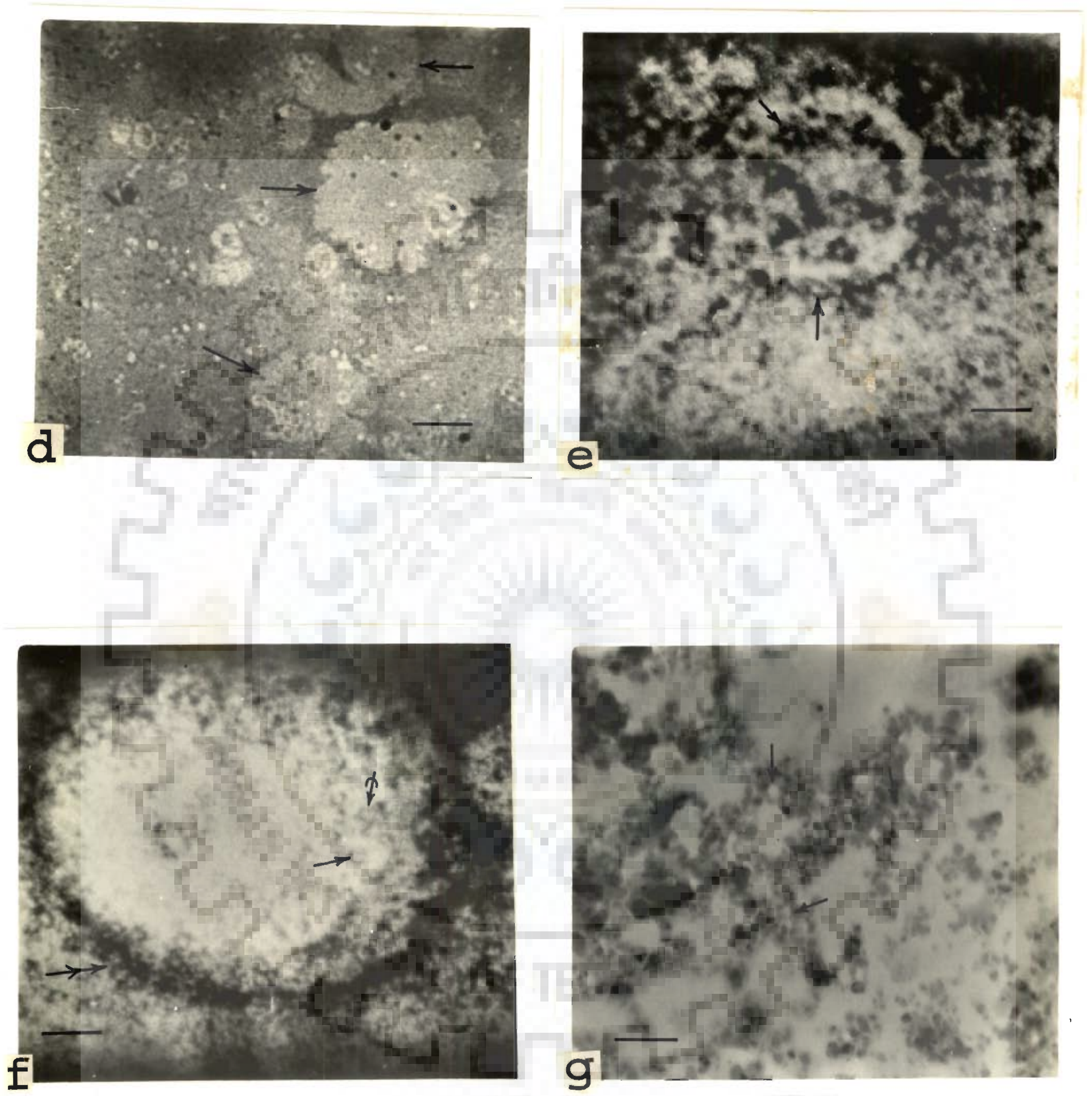


FIG. 22 d - g

CHAPTER 5

5.0 DISCUSSION

This thesis incorporates the results of the studies on isolation, purification and characterization of tonoplast ATPase obtained from 7 d old peanut seedlings. For isolation of tonoplast membrane-enriched fraction, plant tissue was mixed with 4 volumes of homogenizing buffer for facilitating proper homogenization.

Differential centrifugation is probably the most commonly used method for isolation of cell organelles from homogenized tissue. Cell organelles whose size and density differ by significant order of magnitude, sediment at different stages of centrifugation depending upon the applied centrifugal force. Centrifugation at 13,000x g for 20 min pellets 90% of the mitochondria (Giannini and Briskin, 1987). The 13,000x g to 80,000x g pellet consists of microsomal membranes, viz. plasma membrane, tonoplast, Golgi apparatus and the endoplasmic reticulum (Poole et al., 1984). The tonoplast enriched fraction is separated from other membrane fractions of the microsomal pellet by isopycnic centrifugation using 3 step sucrose gradient (Macri and Vianello, 1987; Sen and Sharma, 1994). Tonoplast membranes are known to have a density ranging from 1.10-1.12 g/cm^3 (Quail, 1979) and are harvested from 15/35% sucrose interface (1.0622/1.1566 g/cm^3).

In legume cotyledons, it has been shown that during germination reserved proteins are catabolized within the protein bodies. In castor bean it has been found that when the protein bodies appear empty, their limiting membranes fuse to form central vacuoles (Nishimura and Beevers, 1978). During seedling growth, in mung bean cotyledons, protein bodies contain numerous vesicles with a cytoplasmic content on the third day. Later in seedling growth, these

vesicles appear empty layer. These empty vesicles are believed to be autophagic vesicles resulting from invaginations of the protein body membrane (Van der Wilden, 1980). Present results on tonoplast ATPase activity versus seedling growth reveal that there is substantial rise in tonoplast ATPase activity only after 3 days of seedling growth.

Hypocotyl contributes the maximum amount of ATPase activity compared to other organs of the seedling. It is quite possible that major transport activity of solutes across the cytosol-vacuole interface takes place in this organ which is reflected by pronounced ATPase activity.

The tonoplast ATPase obtained from 7 d old peanut seedlings is similar in several characteristics to the enzyme from other plants. Peanut enzyme is inhibited by the anion NO_3^-, a well established inhibitor of tonoplast ATPase (Randall and Sze, 1986; O'Neill et al., 1983; Smith et al., 1984a) and is insensitive to vanadate and azide, a characteristic of inhibitor sensitivity of the tonoplast ATPase (Marin, 1983). Vanadate is known to inhibit plasmalemma ATPase by blocking the formation of phosphoenzyme intermediate (Wingstrand and Linderberg, 1982). In the present case, greater degree of inhibition of the ATPase associated with high density membranes (35/45% interface) (fraction III) by vanadate suggests their enrichment in plasmalemma. Phloridzin does not inhibit the tonoplast ATPase. Since phloridzin is established as an inhibitor of chloroplast ATPase (Winget et al., 1969), this result indicates lack of contamination by thylakoid membranes. This is understandable since dark grown seedlings are unlikely to develop chloroplasts in them. It has been reported that azide inhibits multisite activity of mitochondrial ATPase in *E. Coli* (Noumi et al., 1987; Gallahar and Leonard, 1982). Low percentage of inhibition of low density membranes (15/35% interface) (fraction II) associated ATPase by azide may indicate very little contamination by inner mitochondrial membranes.

The results reveal a sharp pH optima of 8.0 for tonoplast ATPase. There is a general

consensus on the pH optima value of the enzyme in the range 7.5 - 8.0 (Smith et al., 1984a; Sze, 1985; Kasamo, 1986) although few early reports have indicated somewhat lower value (Lin et al., 1977; Doll et al., 1979; O'Neill et al., 1983). A sharp decline in tonoplast ATPase activity at lower pH (<7.5) may be attributed to very little contamination by plasma membrane ATPase which has a lower pH optima (Briskin and Poole, 1983; Kasamo, 1986).

The K_m (Mg-ATP) value for the tonoplast ATPase is generally reported to be less than 0.5 mM (Sze, 1984; Struve et al., 1985) and this appears to be true for peanut enzyme also. However some workers have reported higher K_m values in other species (Kasamo, 1986; Jochem and Luttge, 1987; Kawata and Yoshida, 1988). Thus there may be some variations in the K_m values of the enzyme from different sources or also perhaps at different developmental stages.

About 50% loss of tonoplast ATPase activity in presence of DDT and ß-mercaptoethanol may indicate significance of dithiol bridges in the active conformation of the enzyme. It is possible that both dithiol bridges and -SH groups are critical for the enzyme activity. Also, it is now established that tonoplast ATPase is inhibited by NEM, mercurials or other compounds that react to -SH groups (Randall and Sze, 1986). Cysteine residues located on 72 kDa appear to be the part of active site of the enzyme (Randall and Sze, 1987b).

Tonoplast ATPase shows a clear preference towards ATP as the substrate in comparison to other nucleoside phosphates. Somewhat higher rates of hydrolysis shown by the enzyme towards other nucleoside triphosphates could be attributed to more structural similarity of ATP with them. This seems to be especially true in case of GTP which is both a purine and a triphosphate like ATP. Tonoplast ATPase has also been shown to catalyze hydrolysis of GTP at higher rates in mung bean (Kasamo, 1986) corn (Mandala and Taiz, 1986) and *Vigna radiata* (Kawata and Yoshida, 1988). However with other nucleoside phosphates as substrates, the rate

of hydrolysis by peanut enzyme is much less.

Divalent metal cation requirement of tonoplast ATPase is well established (Sze, 1985) with Mg^{2+} being the most optimal. Other divalent cations are unable to completely substitute Mg^{2+}. In absence of KCl, Mn^{2+} is able to completely replace Mg^{2+}. But in presence of KCl, Mn^{2+} shows only three fourth of the stimulation that is obtained by Mg^{2+}. Similar result has been witnessed in beet vacuoles (Walker and Leigh, 1981a). It appears that the presence of monovalent ions influences the effect of divalent cations on enzyme activity. In presence of KCl when both Mg^{2+} and Ca^{2+} are present, the ATPase activity is less than when Mg^{2+} is present alone. It is known that Mg^{2+} and Ca^{2+} ions interact with each other during absorption. Moore et al. (1961) have found in barley and maize root that Mg^{2+} uptake is affected by Ca^{2+} and uptake is greater from solution of Mg^{2+} alone than Ca^{2+} is present. On the contrary in the absence of KCl, ATPase activity is stimulated greatly when both Mg^{2+} and Ca^{2+} are present. Though it is difficult to explain the above phenomenon, it is known that Mg^{2+} and Ca^{2+} ions interact with each other during their absorption and this interaction is influenced by the presence of K^+ (Leggett and Gilbert, 1969).

Anion-stimulation of tonoplast ATPase has been reported in several cases (Randall and Sze, 1986; Smith et al., 1984b). Peanut enzyme is also greatly stimulated by Cl^- ions. Although the exact mechanism of action by Cl^- ions is not clear, it has been suggested that these may act as permeant anions and relieve the electrical gradient generated by tonoplast ATPase in the vesicles and thus collapse of the electrical gradient permits more H^+ pumping by the ATPase resulting in formation of a steep pH gradient (Churchill and Sze, 1983; Blumwald and Poole, 1985). Churchill and Sze (1984) have suggested that chloride directly stimulates ATP hydrolysis independent of the electrochemical gradient based on several observations: i) chloride stimulates

ATPase activity even in the presence of ionophores that dissipate the electrical potential and ii) detergent-solubilized ATPase is stimulated by chloride. Schumaker and Sze (1987) have also suggested that in the presence of ATP, Cl^- enhances pH gradient formation, because it stimulates ATPase activity and the inwardly directed H^+ pump is faster than the rate of Cl^- dependent H^+ efflux. In general, anions stimulate ATPase activity with the sequence: $Cl^- > Br^- > I^-$. Peanut tonoplast ATPase is completely inhibited by ClO_3^-, the reason may be that these ions behave as linear uncompetitive inhibitor with respect to Mg-ATP (Farley, 1976). Another possibility of inhibition of tonoplast ATPase activity by ClO_3^- is its reduction to ClO_2^- following coupled transport with H^+ as ClO_2^- is toxic to the plant cells (Chodera and Briskin, 1990). Citric acid is one of the most important acids in plants and it is accumulated in vacuoles (Buser-Suter et al., 1982). But present data shows complete inhibition of tonoplast ATPase activity by citrate. The possible reason might be that citrate transport into the tonoplast is not energized by proton pump. This result is in agreement with that of Oleski (1987b), who could not show any dependence of citrate uptake on Mg-ATP and is in contrast to those of Marin et al. (1981a) and Rentsch and Martinoia (1991). Cation stimulation of ATPase activity can also be explained on the basis of the evidence that tonoplast ATPase activity is regulated by electrochemical gradient (Sze, 1984). The factors that cause dissipation of the gradient will stimulate enzyme activity (Churchill and Sze, 1983, 1984). The difference in stimulation of ATPase activity by the monovalent cations is due to the differential permeability of the membrane to the monovalent cations. In beet root vacuoles (Walker and Leigh, 1981a) it has been shown that differential cation stimulation can be abolished by 0.01% Triton X-100.

Proton uptake by tonoplast vesicles was determined with AO as pH probe. Initial rate of fluorescence quenching is due to activity of the ATPase. Nitrate greatly decreases the initial rate

of fluorescence quenching initiated by ATP and dissipates the pH gradient during the steady state. These results reflect the inhibition of the ATPase. Nitrate had a biphasic effect on proton gradient, causing first an increase and later a decrease in proton gradient. This result suggests that nitrate enters the vesicles and dissipates the membrane potential which results in an increase in H^+-ATPase activity (Blumwald and Poole, 1985). This increase in ATPase activity may account for the initial effect of nitrate in increasing proton gradient. The subsequent dissipation of proton gradient may result from the efflux of nitrate together with protons through a nitrate/proton symport mechanism (Blumwald and Poole, 1985). Whereas when the tonoplast enriched fractions were preequilibrated with nitrate before activation of ATPase with ATP, there is no evidence of biphasic effect on proton gradient but initial rate of fluorescence was inhibited. However, according to Pope and Leigh (1988) AO and NO_3^- form a lipophilic ion pair within the vesicles and this ion pair diffuse across the membrane and increase the permeability of membranes to H^+ thus discharging the pH gradient.

Peanut enzyme was solubilized using Triton X-100. This report is in contrast to the work of Mandala and Taiz (1986) who have found that Triton X-100 is not suitable for solubilizing corn coleoptile ATPase. They have found deoxycholate and octylglucoside as the most useful detergents. However oat ATPase could be solubilized by Triton X-100 (Randall and Sze, 1986). Percentage of Triton X-100, used for solubilization of the enzyme differs from plant to plant. Randall and Sze (1986) have used 5% Triton X-100 in corn root, whereas Parry et al. (1989) and Hosaka et al. (1994) have used 4% and 2% Triton X-100 to solubilize the tonoplast ATPase of beet root and pear fruit respectively. In peanut 5% Triton X-100 is used to solubilize the enzyme. This may point to variations in the topography of the enzyme bound to membranes in different species. The addition of phospholipids stimulate tonoplast ATPase activity of peanut

seedling, when Triton X-100 is used to solubilize the enzyme. In oat root (Randall and Sze, 1986) also phospholipid is found to be essential for reactivation of solubilized enzyme. On the contrary the tonoplast ATPase does not appear to have a lipid requirement in corn coleoptiles (Mandala and Taiz, 1985) when octylglucoside is used to solubilize the enzyme.

Tonoplast ATPase of peanut is purified 27-fold by gel filtration chromatography using Sepharose CL-6B. Lai et al. (1988) and Randall and Sze (1986) have used Sepharose CL-6B and Sepharose 4B respectively for gel filtration during purification of the tonoplast enzyme. However, Mandala and Taiz (1985) have purified the enzyme by high-speed centrifugation in a linear sucrose gradient. They have found that gel filtration and ion-exchange chromatography are not useful tools for purifying the enzyme.

Since it is known that one micelle of Triton X-100 is of 90 kDa (Furth, 1980), the molecular mass of ATPase is estimated in the range from 400 to 600 kDa. Similar results have been obtained in other species like oat (Randall and Sze, 1986), mung bean (Wang et al., 1989). Mandala and Taiz (1985) have reported that the tonoplast ATPase is composed of several different polypeptides as found by the number of bands on SDS gel. On Coomassie-stained gels they have found that the only bands visible from peak ATPase fraction are 72 and 62 kDa. Whereas using silver staining they have reported that many bands are found to be correlated with ATPase activity. Similar observation has been reported in *Neurospora* (Bowman and Bowman, 1985) where a prominent band at 70,000 and other bands at 100,000, 60,000 and 16,000 are associated with the peak of ATPase activity. Randall and Sze (1986) have found that the purified tonoplast ATPase consists of at least three polypeptides of 72, 60 and 16 kDa. Minor components of 40 kDa and 13 kDa are also copurified with ATPase activity. Randall and Sze (1987b) have reported that 72 kDa subunit contains the catalytic site and 60 kDa subunit contains

an ATP or nucleotide binding site, whereas 16 kDa polypeptide functions as proton conductance. They have also found that 72, 60 and 40 kDa are peripheral proteins while 16 and 13 kDa polypeptides are integral proteins. Based on the result of analysis of sucrose gradient fraction on SDS-PAGE, Wang et al. (1989) have reported two prominent bands at 78 kDa and 64 kDa, concomitantly with ATPase activity. However the values are slightly higher than that obtained from the vacuolar ATPase of other higher plants. In peanut, purified tonoplast enzyme fractions, eluted from Sepharose CL-6B column, when analyzed in gradient gel under dissociated condition, are found to consist of several polypeptides. Three major polypeptides, 69, 55 and 20 kDa are purified with ATPase activity and along with them two minor bands of 37 and 15 kDa are also copurified. It might be true for peanut enzyme that 69 and 55 kDa polypeptides constitute the catalytic subunits whereas 20 kDa polypeptide is embedded in the tonoplast membrane and forms proton channel. Parry et al. (1989) have found that though purified tonoplast enzyme constitutes two polypeptides of 67 and 55 kDa, polypeptides with apparent molecular masses of 100, 44 and 32 kDa could be seen in the peak ATPase fractions as minor components. Recently Hosaka et al. (1994) have reported that purified tonoplast ATPase of pear fruit reveals 10 protein bands with apparent Mr of 68, 54, 44, 42, 35, 30, 27, 16, 13 and 12 kDa in SDS-PAGE analysis.

It is well known that the plasma membrane is the primary site of auxin action (Scherer, 1984a,b). Auxin affects cell wall loosening via proton extrusion which is an important step in auxin induced acid growth (Cleland and Rayle, 1978). Proton translocation by plasma membrane H^+-ATPase is either stimulated or inhibited by auxins according to the hormone concentration (Santoni et al., 1990; Szponarski et al., 1991). Higher concentrations inhibit ATPase activity coupled with proton translocation whereas lower concentrations stimulate ATPase activity. Role

of auxins on proton transport at tonoplast is not known.

In vitro response most likely represents primary action of the hormone (Scherer, 1984a). In peanut seedlings IAA does not stimulate tonoplast ATPase activity. Conversely, its effect on ATPase activity is inhibitory. At higher concentration, inhibition by auxin is more compared to lower concentration. IBA and NAA also do not show any stimulatory effect on the enzyme activity. However, pronounced stimulation of ATPase activity has been resulted in presence of PAA. This indicates that the uptake carrier shows greater affinity for PAA than the other auxins.

In *in vivo* experiments none of the auxins is stimulatory to tonoplast ATPase activity. Thus in general it can be said that the ATPases bound to plasmalemma and vacuolar membranes behave quite differently in response to treatment with auxins. In both *in vivo* and *in vitro* experiments, kinetin is inhibitory to tonoplast ATPase activity. Though kinetin is known to stimulate several enzymes, decline in activities of ribonuclease and enzymes of the hexose monophosphate shunt have also been reported (Gupta, 1982). In contrast GA_3 treatment does not result in any significant effect on ATPase activity either *in vivo* or *in vitro* experiments. In *in vitro* experiments, ABA does not show any significant inhibition. The reason may be that the ABA is transported through H^+ ion. At pH 8.0, Baier and Hartung (1988) have found that more than 99.9% of the ABA is present as ABA^-, therefore ABA^- may also be translocated by the substrate transport system across the tonoplast. In *in vivo* experiments, inhibition by ABA is much more pronounced. ABA inhibits proton extrusion and K^+ uptake that occurs during early stages of seed germination and thus inhibits turgor regulated cell growth (Schubert and Matzke, 1985). This may be the reason that in *in vivo* experiments, ABA treated seedlings show 55% inhibition of ATPase activity.

The secondary metabolites particularly phenolic compounds are frequently accumulated

in plants as glycosides (Hosel, 1981). Glycosylated secondary products differ from the free aglycone in two main chemical properties; they show enhanced water solubility and decreased chemical reactivity. Because of these properties, glycosylated compounds are better stored within plant vacuoles and are less reactive toward other cellular components than the aglycones. Hydrolysis of secondary plant glycosides releases the physiologically active aglycones. Accumulation of phenolic compounds have been described in vacuoles of grape berry (Moskowitz et al., 1981), horseradish root (Grob and Matile, 1980). Alibert et al. (1982) have found that sweet clover contains large amount of o-coumaric acid glucosides. It is also known that the most of the higher plants are able to glycosylate foreign phenols (Hosel, 1981). However, site of glycosylation is not known clearly. It has been reported that glycosyl transferases may be bound to endoplasmic reticulum (Conder and Lord, 1983) or located at the tonoplast (Alibert et al., 1982). In the present study, it is found that marked increase in ATPase activity. occurrs when phenolic acids like trans cinnamic acid and phenylacetic acid are added to the assay medium. It is possible that glycosylation takes place in membranes of fraction II and the transport of glycosylated phenolic compounds is facilitated. In the presence of other phenolic compounds, hydroquinone and hydroxybenzoic acid, ATPase activity does not show any stimulatory effect. *In vivo* application of phenolic compounds cause retardation of growth and activity of tonoplast ATPase could not be detected. It is not known, why chlorogenic acid completely inhibits ATPase activity. However, chlorogenic acid is known to inhibit IAA-oxidase (Gortner and Kent, 1958). However its mechanism of action has not been worked out. It is possible that chlorogenic acid completely inactivates tonoplast ATPase by binding to the enzyme directly and altering its conformation.

Tonoplast membrane fractions prepared in sucrose gradient for electron microscopic

studies were nearly free from contamination of other membrane fractions. In presence of azide, membrane fraction II shows only 9% inhibition, whereas azide inhibits 90% of mitochondrial ATPase activity. Absence of plasma membrane from the tonoplast enriched fraction has been verified both from the lack of vanadate inhibited ATPase (4% inhibition by vanadate) and paucity vesicle staining by phosphotungstic acid as at low pH it is regarded as plasma membrane specific stain (Ronald et al., 1972). Stalked particles of the vesicles are similar to those exhibited by *Neurospora* (Bowman et al., 1989), soybean (Moore et al., 1991) and carrot (Taiz and Taiz, 1990). Though the stalked particles of tonoplast resemble that of mitochondria, they differ in size, shape and behaviour. The cleft or apical notch is the main feature of the head piece of tonoplast ATPase but the mitochondrial ATPase lacks the cleft. Unlike tonoplast ATPase, mitochondrial ATPase structure does not disappear from the surface of the vesicles when treated with KNO_3. During electron microscopic study it has been found that all of the vesicles do not show head and stalk structure. The reason may be that the membrane fractions are the mixed population of inside out and right side out vesicles amongst which only the right side out vesicles are capable of exhibiting enzyme structure on the surface of the vesicles (Morre et al., 1991). The second possibility to account the absence of particles on many vesicles is that during isolation and staining procedure many head and stalked structures were lost. Biochemical assay of pellet and supernatant collected after different stages of differential and isopycnic centrifugation (13,000x g pellet and supernatant, 80,000x g microsomal pellet and supernatant, fraction II) (Table 2) supports the loss of enzyme structure. It is found that besides microsomal pellet, 80,000x g supernatant, which is discarded, also shows some nitrate sensitive ATPase activity. Electron microscopic studies also have exhibited that the particles on the surface are sometime discrete and very few are attached to the edges of membrane vesicles. Further

evidence that densities of the particles differ significantly from vesicle to vesicle indicates the possible causes of dissociation of particles which were found lying on the grid adjacent to the membrane. Klink and Luttge (1991) have used methylamine tungstate for negative staining to improve the staining condition. They have reported that vesicles, negatively stained with methylamine tungstate, are associated with large number of head and stalk structures at the edge.

CHAPTER 6

6.0 SUMMARY

Tonoplast membranes possess a proton pumping ATPase. H^+-ATPase generates proton gradient converting the free energy released by the hydrolysis of ATP. Resulting pH gradient acts as the driving force in intracellular transport at the vacuole-cytosol interface.

ATPase associated tonoplast membrane fractions were obtained from 7 d old peanut seedlings using differential and 3-step sucrose gradient centrifugation. Step gradient centrifugation resulted in separation of three membrane fractions, referred as fraction I, II and III. The Fraction II was regarded as tonoplast enriched fraction as the enzyme activity of it was inhibited (45%) by NO_3^- (50 mM). Moreover the density of the tonoplast membrane (1.10-1.12 g/cm^3) is similar to the density range of the sucrose interface (15/35%) (1.0622/1.1566 g/cm^3) where from fraction II was collected. It was also found that fraction II was not significantly contaminated by other membrane fractions. This fraction was used for purification and characterization of tonoplast ATPase.

The enzyme activity was found to be maximum on 7th day.

Organwise distribution of tonoplast ATPase activity showed that though the enzyme was found in all the parts of a seedling, hypocotyl contributed maximum amount of activity.

Tonoplast ATPase exhibited a sharp pH optima at 8.0. K_m and V_{max} values of the enzymes determined by Lineweaver and Burk plot with Mg^{2+} ATP as substrate were found to be 0.15 mM and 3.1 μmol Pi mg^{-1} protein h^{-1}, respectively.

Addition of both dithiothreitol (3 mM) and ß-mercaptoethanol (15 mM) to the assay medium resulted in 45 and 35% loss of ATPase activity, respectively. Addition of EDTA (5.

10, 15 mM) resulted in about 90% loss of ATPase activity.

In the presence of KCl, the order of effectiveness of divalent cations for the enzyme was Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}. However, in the absence of KCl, this order was changed, Mn^{2+} being optimum. Then, compared with Mg^{2+} alone, there was about 2.5 fold higher enzyme activity with both Ca^{2+} and Mg^{2+}.

Peanut enzyme was stimulated about 3-fold by addition of Cl^-. Further with the exception of RbCl, addition of monovalent cations caused about 2-fold stimulation in ATPase activity. The order of effectiveness of monovalent cations on the ATPase activity was NH_4Cl > KCl > NaCl > LiCl, CsCl > RbCl. Amongst organic anions 63% enzyme activity was retained in presence of tartarate than the Cl^- ions control. Citrate caused complete inhibition of activity.

Amongst various nucleoside phosphates, ATP was the most effective for the hydrolysis reaction. GTP was also partially hydrolysed (51%).

Proton uptake by tonoplast vesicles was determined with acridine orange as pH probe. The proton pumping in tonoplast vesicles was inhibited (53%) by addition of 50 mM nitrate.

The enzyme was solubilized using 5% Triton X-100 which was found to be most effective compared to CHAPS, Brij-35 and sodium deoxycholate. 93% of the total ATPase activity was solubilized. Phospholipid was essential for the recovery of the tonoplast ATPase activity in presence of Triton X-100. Optimum phospholipid concentrations, determined for Triton solubilized and gel filtered tonoplast ATPase, were 0.2 mg/ml and 0.05 mg/ml respectively. Centrifugation of microsomal pellets on sucrose gradient, Triton solubilization and subsequent gel filtration (using Sepharose CL-6B column) resulted in about 1.7-, 1.7-, and 27-fold purification of enzyme over the microsomal pellet, respectively. The purified enzyme was highly unstable and lost its activity within 24 h at both 20° and 4°C. By contrast, step gradient

purified enzyme was fairly stable and the activity was retained upto nearly 6 weeks at 4°C. The Mr of ATPase was estimated in the range from 400,000 to 600,000 using reference proteins - thyroglobulin and apoferretin.

SDS-PAGE resulted in separation of three major polypeptides of 69, 55 and 20 kDa. In addition, minor bands of 37 and 15 kDa were co-purified.

In *in vitro* experiment plant hormones like IAA, IBA, NAA, GA_3 and kinetin showed inhibitory effect on the enzyme activity. PAA at all concentrations enhanced the tonoplast ATPase activity by 1.7-1.8 fold. Tonoplast ATPase showed either little or no inhibition in presence of ABA. In *in vivo*, all the hormones except GA_3 showed inhibitory effect.

In *in vitro* experiment, amongst phenolic compounds, chlorogenic acid showed complete inhibition at all concentrations. Hydroquinone and hydroxybenzoic acid, at higher concentration (10^{-3} M) inhibited enzyme activity completely; at lower concentration inhibition decreased. Application of cinnamic acid enhanced the enzyme activity by 1.2-1.8 fold. In *in vivo* experiment, seeds were unable to germinate in presence of phenolic compounds. Only seedlings treated with 10^{-4} and 10^{-5} M concentrations of hydroquinone showed little growth and 10% enzyme activity was determined.

Ultrastructure of the tonoplast ATPase was observed under transmission electron microscopes by the help of negative staining (1% phosphotungstic acid). Tonoplast ATPase was found to be attached to the surfaces of the vesicles which was evident by the presence of head and stalk structure with a distinct cleft or notch in the head portion. This structure disappeared from the vesicle surface in presence of 100 mM KNO_3. However, compared to the tonoplast ATPase, the mitochondrial ATPase lacked the cleft and was shorter in size. No visible effect on mitochondrial ATPase was seen in presence of nitrate.

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