BIOCHEMICAL INVESTIGATIONS ON PLANT MEMBRANES OF STORAGE TISSUES : PURIFICATION AND CHARACTERIZATION OF A GLYCOPROTEIN ACID PHOSPHATASE FROM MEMBRANES OF *PISUM SATIVUM* COTYLEDONS

THESIS

submitted to the University of Roorkee
for the award of the degree
of
DOCTOR OF PHILOSOPHY
in
BIOCHEMISTRY

By

OMAR MOHAMMAD BASBAA

DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY
UNIVERSITY OF ROORKEE
ROORKEE-247 667 (INDIA)

JUNE, 1989

Gratis

FORM F

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"BIOCHEMICAL INVESTIGATIONS ON PLANT MEMBRANES OF STORAGE TISSUES: PURIFICATION AND CHARACTERIZATION OF A GLYCOPROTEIN ACID PHOSPHATASE FROM MEMBRANES OF PISUM SATIVUM COTYLEDONS"** in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy submitted in the Department of **BIOSCIENCES & BIOTECHNOLOGY** of the University is an authentic record of my own work carried out during a period from August 1985 to May 1989 under the supervision of Prof.C.B.Sharma and Dr. Vinay Sharma.

The matter embodied in this thesis has not been submitted by me for the award of any other Degree.

O.M. Bakhoo

Candidate's Signature

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

C. B. Sharma V. Sharma

Date: 21.6.1989

Signature(s) of Supervisor(s)

The candidate has passed the viva-voce examination held on at . The thesis is recommended for award of the Ph.D. Degree.

Signatur of Guide(s)

Signature of External Examiner(s)

..

BIOCHEMICAL INVESTIGATIONS ON PLANT MEMBRANES OF STORAGE TISSUES : PURIFICATION AND CHARACTERIZATION OF A GLYCOPROTEIN ACID PHOSPHATASE FROM MEMBRANES OF *PISUM SATIVUM* COTYLEDONS

ABSTRACT

Various endomembrane fractions have been isolated from 18 h imbibed pea cotyledons by combination of differential and sucrose density gradient centrifugations. Without Mg^{2+} and in the presence of 3 mM EDTA, organelles with average densities of 1.17 $g.cm^{-3}$, 1.15 $g.cm^{-3}$ and 1.10 $g.cm^{-3}$ have been identified as PM, GA and ER on the basis of their characteristic sedimentation properties and enrichment of the marker enzymes for PM (glucan synthase II), for GA (inosine diphosphatase, ID-Pase, and glucan synthase I) and for ER (mannosyltransferase, MTase), in the respective fractions. The PM, GA and ER thus obtained were enriched 12.8, 7 and 8 folds in their specific marker enzymes relative to the crude microsomal fraction (12,000-105,000 xg pellet) and were free of cross contamination as judged by the presence/absence of marker enzymes specific for different endomembranes.

About 10 percent of the total acid phosphatase, APase, (E.C.3.1, 3.2) activity in the 18h imbibed pea cotyledons was associated with the microsomes which were almost completely devoid of 5'-nucleotidases and hexose phosphatases. Of the total microsomal APase activity, about 35, 4.5 and 6.4 percent was associated with the PM, GA and ER respectively.

p-nitrophenyl phosphate (pNPP) was the best substrate for the membrane-bound enzymes from PM, GA and ER followed by ATP which was hydrolysed to the extent of 53, 61 and 74 percent relative to pNPP, by PM-, GA-, and ER-APase respectively. Phosphorylated sugars and nucleoside monophosphates were only slightly hydrolysed by the membrane-bound APase particularly the PM-bound. The apparent Km values of PM-, GA-, and ER- associated enzymes using pNPP as substrate were 500 µM, 500 µM and 310 µM respectively. Their Vmax values were 66.7, 28.6 and 33.3 µM per min per mg protein, respectively. The PM-, GA-, and ER-associated APases exhibited identical optimum pH range of 5.25 - 5.75, above and below which the activity of the enzymes declined sharply.

The PM-, GA- and ER-APases were activated 25, 15 and 18 percent by EDTA. Citrate activated the PM-, GA-, and ER-associated enzymes to 426, 140 and 233 percent respectively whereas tartarate stimulated these enzymes to the extent of 375, 126 and 220 percent in order. Na^+ and K^+ were without effect. Most of the divalent metal ions tested (Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, 10mM) were inhibitory to the PM-, GA-, and ER-APases, although the potency of inhibition varied markedly. Zn^{2+}, Mn^{2+}, Hg^{2+} and Cu^{2+} were highly potent inhibitors. Ni^{2+}, Ca^{2+} and Mn^{2+} had differential inhibitory effect on these membrane-bound enzymes. While there was little inhibition of the PM-APase by a Ca^{2+}, the GA- and ER-APase were inhibited by 43 and 67 percent respectively. Ni^{2+} inhibited the PM-, GA-, and ER-APase by 83, 43 and 63.5 percent respectively, whereas Mg^{2+}

inhibited these enzymes by 36, 55 and 58, in order. Pi, F^- and $Mo_7O_{24}^{6-}$ were strong inhibitors of the three membrane-bound APases.

The PM-bound APase was stable for one month at 0-4^oC whereas the GA-, and ER-APases were rendered totally inactive in one week. However, about 80 percent of activity was retained upto 3 days storage at the same conditions.

Apart from their difference in their stabilities, the membrane-bound enzymes were remarkably similar with respect to pH optimum, metal ion effect and response to inhibitors and activators.

A major acid phosphatase was purified to homogeneity from the PM of pea cotyledons by selective solubilization of the enzyme with 1 percent CHAPS at a protein-to-detergent ratio of 2:3 in the presence of 5mM EDTA, followed by ion exchange chromatography on DEAE-Sephadex, acid precipitation at pH 5.0 and CM-Sephadex column chromatography. Both native and SDS-PAGE of the enzyme revealed the presence of a single polypeptide chain of around 68 kD molecular weight, though molecular weight by gel filtratioon was found to be 69,000 - 70,000 daltons. The purified enzyme was highly unstable losing total activity in 3 days at 0 - 4^oC and after one week at - 20^oC.

The purified PM-APase exhibited maximal activity between 5.2 to 5.6 pH range and mximal stability over a pH range of 4.8 to 6.4. The Km and Vmax values for pNPP as substrate were 3.1 x 10^{-4} M and 2 mM per min per mg protein, respectively. Inorganic

phosphate (Pi) and fluoride inhibited the enzyme in a competitive and noncompetitive manner, respectively. The Ki value for Pi was found to be 0.4 mM. Besides pNPP, the PM-APase also hydrolysed nucleoside di and nucleoside triphosphates but with only 30-40 percent efficiency. Nucleoside monophophates and phosphorylated sugars were not hydrolysed.

The enzyme was unaffected by citrate or tartrate, contrary to the PM-bound enzyme to which they were strong activators. Metal ions were not required for activity. Mg^{2+} and Ca^{2+} showed only slight inhibition while Mn^{2+} and Ni^{2+} inhibited the enzyme to about 33 and 60%, respectively. Zn^{2+}, Hg^{2+}, Cu^{2+}, F^{-} and molybdate were strong inhibitors, inhibiting the enzyme activity almost totally. Na^{+} and K^{+} were without effect.

The PM-APase was found to be a glycoprotein with 21.1% carbohydrate. Digestion of the enzyme with endo-N-acetyl- β-D-glucosaminidase H (endo-H) released about 70 percent of the total carbohydrate content of the enzyme indicating that the oligosaccharide moiety was asparagine-linked high mannose type. Both periodate treatment of the enzyme and deglycosylation by endo-H resulted in loss of enzyme activity indicating the essential role of the carbohydrate moeity for the activity of enzyme.

The Golgi apparatus acid phosphatase was also purified to homogeneity using essentially the same purification scheme used for the purification of the PM-APase except that gel filtration

was introduced in the purification procedure in place of DEAE-Sephadex.

The molecular weight of the GA-APase was 65,500 daltons by gel filtration and 61,100 daltons by SDS-PAGE. The pH optimum of the enzyme was in the range of pH 5.2 to 5.6. Km and Vmax values were 3.6×10^{-4} M and 0.87 mM/min/mg protein, respectively. The enzyme was most active towards pNPP followed by nucleoside triphosphates (ca 35%). Nucleoside diphosphates were hydrolysed only slightly (10-15 percent) whereas 5´-mononucleotides and phosphorylated sugars were not hydrolysed at all. The enzyme did not require metal ions for activity. Ca^{2}, Mg^{2+}, and Mn^{2+} inhibited the enzyme from 20 to 30 percent. Hg^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} were potent inhibitors, inhibiting 100, 80, 62 and 82 percent of the enzyme activity, respectively. F^{-}, PO_4^{3-} and $Mo_7O_{24}^{6-}$ were also strong inhibitors. The mode of inhibition by fluoride and orthophosphate was noncompetitive and competitive, respectively. The enzyme activity was not stimulated by citrate and tartarate. The enzyme was a glycoprotein containing about 19.1 percent carbohydrate. Endo-H treatment released about 50 percent of the carbohydrate in 20 h indicating that the enzyme was linked to the asparagine residue of the peptide chain through N-glycosidic linkage and was of a high mannose type. The GA-APase was also senistive to endo-H and periodate treatments, although the sensitivity was relatively smaller than the PM-APase. However, the results indicated the presence of N-glycosidically linked oligosaccharides and the requirement of the carbohydrate for the enzyme activity of the GA-APase.

Based on the close resemblance between the PM-APase and GA-APase, it is suggested that the GA-APase may be a precursor of PM-APase in pea cotyledon cells and that it may be used as a model glycoprotein enzyme for studying the intracellular transport of proteins that are destined to become PM associated proteins in pea cotyledon cells.

ACKNOWLEDGEMENT

I would like to heartily express my deep gratitude and indebtedness to Dr. C.B.Sharma (Ph.D. Texas), Professor, and Dr. Vinay Sharma, Lecturer, Department of Biosciences and Biotechnology, for their excellent guidance, constant help and collaboration during the conduct of this research work.

I acknowledge Head of the Department and the rest of the staff for providing the necessary research facilities.

I also acknowledge University of Aden, People's Democratic Republic of Yemen (P.D.R.Y.) for offering this scholarship under Indo-P.D.R.Y. Cultural Exchange Programme, and to the Department of Education, Ministry of Human Resources Development, Government of India, for the financial support.

My thanks are due to Dr. S.P.Srivastava, University of Aden (P.D.R.Y.), Dr.V.Charan, Mech. Engg. Deptt., Dr. D.R.Gupta and Dr. Kamaluddin, Chemistry Deptt., U.O.R, for their kind assistance during my stay in India.

I also thank my colleagues and research scholars as well as my Indian friends particularly Jaba Das (Mrs.), Ujjwalla Kamat (Miss) and Mr. Sunil Sharma for their benignity, assistance and collaboration.

I feel deeply thankful to my friends at home who flooded me with letters fraught with goodwill and encouragement.

Finally I am sincerely grateful to my mother, wife and the rest of my family, for their blessings, tolerance, and patience.

O.M. Basbaa

OMAR MOHAMMAD BASBAA

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine 5´-monophosphate
5´-AMPase	5´-adenosine monophosphatase
APase	Acid phosphatase (EC 3.1.3.2)
Asn	Asparagine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bis	N, N´-bis-methylene acrylamide
CDP	Cytidine diphosphate
CHAPS	(3 - [(3 - cholamidopropyl) - dimethyl-ammonio] - 1 - propane sulfonate
CMP	Cytidine monophosphate
Con A	Concanavalin A
CTP	Cytidine triphosphate
EDTA	Ethylenediamine tetraacetate
ER	Endoplasmic reticulum
ER-APase	Endoplasmic reticulum acid phosphatase
GA	Golgi apparatus
GA-APase	Golgi apparatus acid phosphatase
Gal	Galactose
GDP	Guanosine disphosphate
GDP - Man	Guanosine diphosphate mannose
Gfw	Gramme fresh weight
Glc	Glucose
Glc-1-P	Glucose-1-Phosphate

Glc-6-P	Glucose-6-Phosphate
GlcNAc	N-acetylglucosamine
Gly-3-P	Glyceraldehyde-3-Phosphate
GMP	Guanosine monophosphate
GTP	Guanosine Triphosphate
IDP	Inosine diphosphate
Km	Michelis-Menten constant
Man	Mannose
pNP	Paranitrophenol
pNPP	Paranitrophenyl phosphate
Pi	Inorganic phosphate
PM	Plasma membrane
PM-APase	Plasma membrane acid phosphatase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
TEMED	N, N, N´, N´-Tetramethylethylenediamide
UDP	Uridine diphosphate
UDP-Glc	Uridine diphosphate glucose
UMP	Uridine monophosphate
UTP	Uridine triphosphate
Vmax	Maximum velocity

1.0 INTRODUCTION

It is generally accepted that in both animal and plant cells the proteins that are destined for secretion or to become part of the plasma membrane (PM) are synthesized in the rough endoplasmic reticulum (RER), vectorially discharged into the lumen of RER, translocated to the Golgi apparatus (GA) and finally packed in secretory vesicles for transport to PM (189). Largely, the information on the intracellular transport of proteins is based on the ultrastructural studies of animal cells and only very few studies have been made with plant cells (12, 54, 68, 79, 103). Moreover, direct biochemical evidence on the intracellular transport of proteins in plants is lacking. Recently, however, Chrispeels (54) has provided first biochemical evidence that a specific storage protein which accumulates in seeds (protein bodies) is modified in, and passed through the Golgi apparatus on its way to the protein bodies. But the data on the PM proteins are lacking.

We have been interested in new models such as plant glycoproteins transported to the cell wall. In these cases we speculate that the intracellular transport uses membrane flow from ER to plasmalemma via Golgi apparatus (171), and thus, several post-translational modifications in glycan structure can be expected(115). However, the plant models using largely storage glycoproteins in legumes and cereals have not revealed processing reactions on the carbohydrate moiety during the intracellular transport (30, 38). But these may be the cases where glycosylation is not essential for secretion of the protein

as in the case of α-amylase in germinating cereal seeds (2, 164, 227) which may not necessarily be passing through the GA (79). On the other hand PM glycoproteins would pass through GA.

Acid phosphatase (E.C. 3.1.3.2) appeared to be a good candidate for studying the intracellular transport of a glycoprotein to the PM and also to study the regulation of the intracellular transport involving glycan moiety for the following reasons :

(i) This enzyme is widely distributed in nature and has been identified in a large variety of organisms and tissues (111).

(ii) It is also ubiquitous in cell membranes and cell walls (17,138).

(iii) In yeast cells it has been used as a marker enzyme (147, 182).

(iv) It is a glycoprotien (17, 81, 138, 182, 192) and its carbohydrate variants also occur (17).

The present study was undertaken to pruify and characterize acid phosphatase from plasma membranes and Golgi apparatus membranes from pea cotyledons and to compare their properties with a view to use them as model proteins for studying the intracellular transport of proteins that are destined to become part of the plasma membrane in plant seeds.

2.0 LITERATURE REVIEW

2.1 Plant membranes

2.1.1 General considerations

In order to investigate any organelle biochemiclly, it must be isolated from the tissue in question in morphologically intact, physiologically active form and as free as possible from cross contaminations. However, the mechanical means employed to break open the cells of tissues to form a homogenate, also disrupt the fragile membraneous organelles such as plasma and tonoplast membranes, endoplasmic reticulum and Golgi bodies (53, 78, 99, 102, 145, 167) into vesicles of microsomes of more or less equal sizes, shapes and densities (Table I). In plants the presence of the hard cellulosic cell wall surrounding the cell is expected to create more serious vesiculization and fragmentation than animals. Any membrane vesicle resulting from tissue homogenization is thus a potential contaminant of the other (78, 198). In addition, break down of the nuclear envelope will release the polybasic proteins that may adsorp to the polyanionic components of membranes and thereby increase the tendency of their aggregation (78). Also the sticky materials released from disrupted nuclei and chloroplasts may entrap other cell particles and membrane vesicles (198). Rupture of the centrally located vacuole of plant cells which contains tannins and phenols (148) and acids and hydrolases (156) creates further problems through alteration of membrane composition, degradation and denaturation of membrane components as well as adsorption. Hence,

TABLE I : Densities and commonly used markers of plant subcelluar components and their derivatives (200)

Subcellular fraction	Density (g/cm^3)	Biochemical marker
Mitochondria (intact)	1.18-1.2	Cytochromecoxidase Fumarase
Mitochondria (outer envelope)	1.1	Antimycin A-sensitive NADH -cytochrome c reductase
Mitochondria (inner membrane)	1.14	Cytochrome c oxidase, succinate dehydrogenase, succinat : cytochrome c reductase
Chloroplasts (intact)	1.21-1.24	Chlorophyll
Chloroplasts (envelopes)	1.12	Galactosyltransferase
Chloroplasts (thylakoid membranes)	1.16-1.18	Chlorophyll
Rough endoplasmic reticulum (RER)	1.15-1.18	RNA
Smooth endoplasmic reticulum (SER)	1.11-1.12	Phospholipid synthesizing enzymes, antimycin A-insensitive NAD(P)H-cytochrome c reductase
Golgi (intact)	1.12-1.15	Latent IDPase, glucan synthase I
Golgi (secretory vesicles)	1.14	Latent IDPase, glucan synthase I
Plasma membrane	1.13-1.18	$Mg^{2+}K^{+}$ - ATPase, glucan synthase II, vanadate sensitive ATPase (18,37, 220)
Vacuole (intact)	1.03 to 1.18	RNAse, phosphodiesterase
Vacuole (tonoplast)	1.1(7)	NO_3- sensitive, vanadate insensitive H^+ - ATPase (63, 64, 186)

a with minor modifications.

TABLE II : Protective agents used in the preparation of plant brei (197)

Agent	Target substance
Cyanide	Heavy metal oxidases
Polyvinylpyrrolidone	Polyphenols, quinones
Ascorbic acid	Quinones
Mercaptoethanol, glutathione, cysteine, Cleland´s reagent, other thiols	Quinones, disulfides, peroxidases
Diethylpyrocarbonate, bentonite, spermidine and other polyamines	Ribonucleases
Serum albumin	Surface denaturation
EDTA	Heavy metals
NH_3 infiltration	H^+
Coconut milk	?

homogenization is carried out in an osmoticum, usually sucrose, containing some protectants (Table II).

Considerable amount of membranes pellet in the initial centrifugation step employed to clear the homogenate and remove the large particles (47, 50, 176).

The cellular origin of the microsomes resulting from homogenization is often difficult to define morphologicallly. Also contaminants from other organelles or vesicles may not be noticed by morphology alone. Hence enzyme markers i.e. enzymes ideally located in only one subcellular organelle, are often employed to identify membranes. However, unequivocal and reliable markers for plant membranes are not available as they occur in more than one membrane (200).

2.1.2 The Plasma membrane

2.1.2.1 Isolation

Recently some excellent reviews on plant plasma membranes have been published (102, 145).

Isolation of plasma membrane has been reported from roots of maize (67, 186, 187) barley (74, 75, 132), soybean (25, 276), beetroot (18, 36, 37, 187, 196), pea (90), mung bean (125), oat (63, 64) and cucumber (158). It has also been prepared from radish seeds (204), leaves of barley (132), suspension cultured carrot cells (203) and rose (117). In most of the cases a post-mitochondrial fraction from 13,000xg for 15 min to 80,000xg for

30 or 35 min (18, 25, 36, 37, 67, 117, 125) or 10,000xg for 20 min to 100,000xg for 30 min (74, 75) is loaded on a continuous or discontinuous gradient and centrifuged for 2 to 3 h at 80,000xg, 100,000xg or 120,000xg. In linear sucrose density gradient the PM bands at a sucrose concentration equivalent to a density range of 1.14 - 1.17 (102, 145, 200). However, variations in its localization in linear sucrose density gradient for the same tissue do exist. For instance, the PM from pea epicotyl has been reported to band at a bouyant density of 1.13 g/cm^3 (195, 236) and 1.18 - 1.19 g/cm^3 (8). A density of 1.173 g/cm^3 has been reported for a PM fraction isolated from developing pea cotyledons (175).

A six-step non-linear sucrose density gradient of 45,38, 34, 30, 25 and 20% is widely used to isolate plasma membrane from plant tissue (109). Recently a 4-step sucrose gradient consisting of 45, 34, 30 and 20% sucrose is described (110). Simplified discontinuous sucrose density gradients are more often used to prepare the PM. A 3-step gradient of 20, 34 and 45% sucrose has been employed to prepare a plasma membrane-rich fraction from soybean roots (276). A plasma membrane enriched fraction from stored beet root has been collected from the interface of 25/30 (36), whereas from fresh roots, it is collected from an interface of 34/40 of a 4-step gradient of 16/24/34/40% sucrose (18) or 34/38% in gradient of 16/24/34/38 percent sucrose (37, 187). Several 3-step gradients have been used to isolate plasma membranes from corn roots (186), pinto

bean leaves (69), soybean roots (25), mung roots (125), rose cell (117) and barley roots (75). A simpler step gradient has bean described for preparation of a PM-membrane rich fraction from corn roots by suspending the microsomal fraction on a 17% (72) or 20% sucrose (73) and layering it on a 34% sucrose.

Dextran gradient rather than sucrose gradient has been used to prepare plasma membranes from oat (63, 64) and pea roots (90). Renograffin or Renograffin and dextran has been employed to isolate a plasma membrane fraction from suspension cultured carrot cells (203).

Aqueous two-polymer phase partition system consisting of dextran and polyethyleneglycol and NaCl has been used to prepare plasma membrane fractions from wide varieties of plant tissues such as oat roots and shoots (277), orchord grass (271), winter rye seedling (259), roots and shoots (260),mulberry bark cells (272), Jerusalem artichoke tubers (118) mung bean hypocotyl (273) and barley roots and leaves (132). In this system the plasma membranes preferentially partition into the upper polyethylene phase. A comparison has recently been drawn between the two-polymer phase system and sucrose and shown to be rather similar (110). Other techniques such as isoelectric focusing (100) and free-flow electrophoresis (220) have been used for the isolation of plasma membranes from plant tissues. Plasma membranes isolated by free-flow electrophoresis are rather similar to those prepared by two-polymer phase system (220).

2.1.2.2 Marker and associated enzymes

Several different markers have been proposed for the identification of the plasma membrane and are frequently used collectively or individually in its studies. These include phosphotungestic acid chromic acid (PTAC) at low pH, N-1-naphthyl ⁀ phthalamic acid binding, glucan synthase II and K^+ - stimulated - mg^{2+} dependent ATPase assayed at pH 6.0 or pH 6.5 (102, 139, 200). Cellulase has been reported to be associated with plasma membranes from abscission zones of Phaseolus vulgaris (129), and pea epicotyl (195) and has been proposed as a marker for the PM (195). More recently vanadate-sensitive K^+-Mg^{2+} - ATPase has been extensively used as a marker for PM (18, 37, 220, 273). However, vanadate is not a specific inhibitor for PM - ATPase as it also inhibits non-specific acid phosphatase (91). Besides it is not absolute for PM as it also inhibits an ATPase from pea chloroplast envelope membranes (160). Uridine diphosphoglucose sterol : glucosyltransferase which transfers glucose to lipid soluble products has been used as a marker for plasma membrane from carrot roots (92), corn coleoptiles (47, 48), pea roots (90), cress roots (40) and soybean hypocotyl (220). However, the enzyme has also been reported to be associated with the Golgi apparatus from Phaseolus aureus (34) and onion stem (146). A blue light induced absorbance change (photoexitation) indicating reduction of a b-type cytochrome has been detected in the plasma membranes of several plant tissues and has been proposed as a marke for PM (139, 277).

Partition of the plasma membrane in the upper polyethyleneglycol phase of the two-polymer phase system as well as its stability against Zn^{2+}, and pH values below 5, have been suggested as markers for the PM (259, 271).

Electron transport components consisting of flavin, cytochrome b-type NADP (H) dehydrogenase as well as NAD(P) ferricyanide reductase, have been reported to occur in plasma membrane fraction isolated from oat roots (202). ATP-dependent calcium transport is reported to be present in plasma membrane isolated from soybean hypocotyl (134). Evidence has recently been presented for the existance of H^+-translocating ATPase with properties similar to the K^+ - ATPase, in the plasma membrane of a variety of plant tissues such as roots of oat, (63, 64), corn (67, 186), pea (90), Phaseolus mung beans (125), soybean (25), beet (18), and barley (75) and radish seeds (204).

2.1.3 Golgi apparatus

2.1.3.1 Isolation

Isolation of Golgi apparatus in a pure intact and active form is difficult to attain as its dictyosomes unstalk into their cisternal components which may further be fragmented into vesicles and may cosediment with other membranes (99, 167). However, use of low shear and inclusion of stabilizers such as coconut oil (170), low concentrations of glutaraldehyde so that the associated enzymes are not inactivated (206), dextran and BSA (99) is recommended to preserve the structure of Golgi apparatus

particularly if electron microscope will be used for identification.

In sucrose density gradient, Golgi apparatus bands at a sucrose concentration of 29 - 33% which is equivalent to 1.12 - 1.15 g/cm^3 (99, 200). Two methods for the isolation of Golgi apparatus enriched fractions have been recently described (99). More recently a Golgi enriched fraction has been isolated from Phaseolus vulgaris cotyledons by passing the 1000 xg supernatant through a Sepharose 4B column to separate the soluble proteins from the organelles. The organelles eluted in the void volume are then applied to a linear 16 - 48% sucrose density gradient and centrifuged at 150,000 xg for two hours (56). Alternatively, the 1000 xg supernatant is layered directly on a discontinuous sucrose gradient of 35 and 16% sucrose and centrifuged for 90 min at 150,000 xg (120). Chanson and his associates have recently isolated Golgi fraction from corn coleoptiles by loading the 1000 xg supernatant on linear and non-linear sucrose density gradients (47, 48, 49). Two fractions of Golgi apparatus have been recently isolated from protoplasts of suspension cultured cells of sycamore by loading the mitochondrial and microsomal fraction on linear sucrose density gradients of 15 - 50% and 10 to 50% and centrifuged at 21,000 xg for 3 hours (6, 7).

2.1.3.2 Marker and associated enzymes

Inosine diphosphatase (IDPase) has been demonstrated biochemically to be associated with the Golgi apparatus (206). However, cytochemical studies reveal that the enzyme is also

localized in the vacuole, endoplasmic reticulum and the cytoplasm (65). Nevertheless, Golgi associated enzyme is characterized by its latency in the sense that profound increase in activity occurs when stored for 2-3 days in the cold (197, 206) and thus distinguished from other activities. It is thus widely used as Golgi marker (6, 7, 47, 48, 49, 56, 220). Another nucleoside disphosphatase, latent uridine diphosphatase (UDPase) has been also used as a marker for Golgi apparatus (47, 48, 177, 247). IDPase is associated with both cisternae and secretory vesicles, while UDPase is associated with secretory vesicles only (247).

Several glycosyltransferases have been detected in Golgi rich fractions though not exclusively (99, 167, 200). The most widely used Golgi marker is glucan synthase I assayed at low UDP-glucose (high km) and high Mg^{2+} (205). It has been used recently to identify Golgi apparatus from various tissues (7, 47, 48, 220, 247). However, minor activity is associated with the PM (47, 211). Galactosyltransferase (UDP - galactose N-acetylglucosamine galactosyltransferase) has been detected in Golgi fraction of onion stem (197) and has been used recently as a marker for Golgi fractions from suspension cultured cells of sycamore (6). Although UDPG sterol:glucosyltransferase has been detected in Golgi apparatus of onion stems (146) and Phaseolus aureus (34) and suggested as a marker for it, its presence in the PM is also reported and used as a marker for the PM (40, 47, 90, 92). Acid phosphatase (7) and α - mannosidase (6) have been used as markers for Golgi fractions from suspension cultured cells of sycamore.

A peak of acid phosphatase has been found to coincide with latent IDPase in sucrose gradient of pea epicotyl and suggested that it is also a marker of Golgi apparatus at least in peas (195).

A mannosyltransferase has been detected in Golgi apparatus of developing pea cotyledons that seems to be not involved in the formation of lipid-linked intermediate utilized in glycoprotein biosynthesis (175). UDPGlcNAc:glycoprotein GlcNAc transferase that transfers GlcNAc residues from UDP-GlcNAc to the oligosaccharide side chain of glycoproteins has been recently demonstrated to be localized in Golgi apparatus of Phaseolus vulgaris (56). UDP-fucose : glycoprotein fucosyltransferase (240) and arabinosyltransferase (92) have been reported to occur in the Golgi apparatus of cucumber and Phaseolus vulgaris respectively. The Golgi apparatus of Phaseolus vulgaris also contains three glycosyltransferases that transfer GlcNAc-fucosyl-, and xylosyl- residues to the oligosaccharide side chain of glycoproteins and are involved in the transformation of high mannose glycoprotein to the complex type (120). Immunocytochemical studies reveal the localization of α-galactosidase hemagglutinin in the Golgi body and protein bodies of developing soybean cotyledons indicating involvement ofGolgi apparatus in intracelluler transport (105).

K^+-Mg^{2+} - ATPase has been reported to occur in the Golgi apparatus of pea stem (206), pea epicotyl (195), corn coleoptiles (47) and suspension cultured cells of sycamore (6). This ATPase has been shown to function as a proton pump (6, 48). The Golgi

apparatus from corn coleoptiles has been demonstrated to possess an inorganic pyrophosphate driven H^+-transport, mediated by a H^+-translocating pyrophosphatase (49).

2.1.4 Endoplasmic reticulum

2.1.4.1 Isolation

The endoplasmic reticulum has been recently reviewed (53, 153). It may be classified into smooth (SER) and rough (RER), the latter is studded with ribosomes. The RER may be deliberately converted into SER by detaching its associated ribosomes with EDTA (15) and thereby decrease its density (Mg-shift or EDTA-shift or density-shift) (200). However, Mg will not allow the ribosomes to dissociate. This density shift is an invaluable tool for the localization of ER in sucrose density gradients. The average density of RER in sucrose gradient is 1.17 g/cm^3 (53) i.e. in the range of 1.15 - 1.18 g/cm^3 (200), but the SER bands at a density range of 1.10 - 1.12 g/cm^3 (53, 153, 200).

Linear sucrose gradients employed in ER isolation vary from 6 to 45 or 53% (274), 15 or 16 to 48 or 50% (1, 262). Centrifugation of the gradient is for 2h at 150,000 xg (1, 262) or 100,000 xg (43). In step gradient the ER is collected from the interface of 26/35 in a 4-step gradient of 35, 26, 23 and 13% sucrose (43), or the interface of 16/35% sucrose in a two-step gradient (1, 262). An ER-enriched fraction from cress roots has been recently prepared by loading the postmitochondrial fraction

(10,000 xg for 30 min) into a discontinuous gradient of 0.6, 1.0, 1.14 and 1.2 M sucrose and collecting the resulting pellet after 45 min or 90 min centrifugation at 90,000 xg (41, 42). Separation of soluble proteins from organelles has recently been used by passing the homogenate though a Sepharose 4B column and collecting the organelles eluted in the column void volume before isolation of the ER on linear or non-linear gradient (1, 30, 55, 262).

2.1.4.2 Marker and associated enzymes

NADH and NADPH-cytochrome c reductases are extensively used as marker enzymes for plant ER (28, 41, 43, 98) which are distinguished from the mitochondrial enzyme by their insensitiveness to antimycin A (151). Choline phosphotransferase (33, 58, 152), and glycoprotein fucosyltransferase (58) have been used as markers for the ER of castor bean endosperm. Glucose-6-phosphatase, a marker for mammalian ER is rarely used as a marker for plant ER (114) as its activity is very low in plant membranes (109). The density shift is frequently utilized to confirm the localization of ER marker enzyme in sucrose density gradients (43, 56, 98, 222, 274).

UDP-N-acetylgucosamine N-acetylglucosaminyltransferase and GDP-mannose mannosyltransferase activities which are involved in the assembly of lipid-linked intermediates, have been detected in the RER of developing pea cotyledons (175). The ER from castor bean endosperm has been subfractionated by centrifugation on a

floatation gradient into two subfractions with uneven glycosyltransferase composition (58). The transferases that catalyze the synthesis of lipid intermediates containing either N-acetylglucosamine or mannose are predominantly located in the upper subfraction, whereas the transferase that catalyzes the transfer of glucose from UDP-glucose to chloroform/methanol-soluble glycolipid is localized in both ER subfractions (58).

Phosphatidylinositol synthase which catalyzes the final step in the de novo synthesis of phosphatidylinositol has been solubilized from the ER of germinating soybean (212). CTP : phosphatidate cystidine 5-diphosphate-diacylglycerol, a central intermediate in the synthesis of phospholipids, is recently characterized from the ER of castor bean endosperm (128). The ER of mung bean cotyledons is associated with two hydrolases (ribonuclease and vicilin peptidohydrolase) which accumulate in the protein bodies during seedling growth, thus indicating involvement of ER in the transport of these enzymes to their site of function (261). α- Mannosidase isozymes which are involved in the processing of the oligosaccharide chain of mannose - containing glycoporteins, are located in the RER, protein bodies and cell wall of Phaseolus vulgaris (262) indicating that they may be synthesized in the RER and transported to their respective destinations-the protein bodies and the cell wall (262).

An ER - enriched fraction from Hippeastrum petal protoplasts has recently been found to be associated with

phenylalanine ammonia lyases, chalcone synthase, glucosyltransferase and trans-cinnamate 4-mono-oxygenase indicating that the ER is a site of phenylpropanoid and flavonoid metabolism in Hippeastrum (274).

Several reserve proteins have been reported to be synthesized on the rough endoplasmic reticulum. The RER of developing Phaseolus vulgaris cotyledons is the site of synthesis of vicilin (28) and phaseolin (30). Rice glutelin is also synthesized on membrane-bound polyribosomes (269). Pea legumin is synthesized on the RER of the developing cotyledons and transported to the protein bodies where it is processed into smaller units (55). Oat globulin polypeptides are synthesized as higher molecular weight precursors on the RER and probably transported into protein bodies where they are processed into smaller subunits (1).

A significant proportion of malate synthase, a glyoxysomal enzyme, is located in the ER (33). The enzyme is synthesized on membrane-bound ribosomes and then discharged into the cisternae of the ER before it is finally sequestered in the glyoxysomes of the developing castor bean endosperm (33). Aggregated forms of malate and citrate synthase are present in the ER of castor bean endosperm during early germination (98). Two serine hydrolases have been detected in the ER of spinach leaves (221) and callus (222, 223). ATP-dependent calcium transport system is detected in the ER of garden cress roots (41, 42), cultured carrot cells (43) and oat roots (226), at least one is Ca, Mg-ATPase (43).

2.2 / Acid posphatases

Acid phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1 3.2) is widely distributed in nature (111). Dormant seeds including peas contain varying amount of acid phosphatase activities which increase after imbibition and during germination, though maximum increase is attained at different period of germination (22, 119, 130, 209). This increase is due to de novo synthesis as it is suppressed by cycloheximide (22,119).

Under phosphorus deficiency, tissue extracts from tomato (20), roots of subterranean clover (70), karri (183) and, roots (243) and leaves of wheat (15, 16) have elevated acid phosphatase activity. In some cases, the increase in phosphatase activity is accompanied by a drop in the phosphate content (70, 15). It has been assumed that the increased acid phosphatase activity in roots may participate in the adaptation mechanism under phosphate deficient condition by hydrolysing organic phosphate in the soil (20, 243). Only one fraction of the phosphatase activity in wheat leaves is increased by the phosphorus deficiency (16). The total acid phosphatase activity in duckweed is increased by 50% under phosphorus starvation and is accompanied by the appearance of two new isoenzymes (207). Depletion of inorganic phosphate enhances the synthesis and release of acid phosphatase in suspension culture of tobacco cells (258). The extracellular acid phosphatase of tobacco cultured cells has been resolved into three fractions by sequential chromatography, two of which are

neutral pyrophosphatases with diesterase activity while the third isoenzyme is the acid phosphatase that is induced by the phosphate deficiency (181). Both tomato plants and suspension cultured cells exhibit phosphate deficiency inducible excretion of acid phosphatase which may be a part of a rescue system to solubilize organic phosphate in the external medium under phosphate deficient condition (96). It is composed of two isoenzymes of low molecular weight that may associate to form high molecular weight aggregates (97). Most of the Pi deficiency induced excreted acid phosphatase is in the low moleculor weight fractions (97).

Acid phosphatases are also affected by other environmental changes. Water stress causes an increase in only one fraction of acid phosphatase in wheat leaves (16). Incremental salt stress also brings about an enhancement of acid phosphatase in spinach leaves which is more specific to the high molecular form (190). Likewise, calcium deficiency causes specific increase in the level of only two of the several phosphatase isoenzymes in cucumber roots (266).

The activity of acid phosphatase and its release is under gibberellic acid control in wheat half-seeds (3, 136), barley (9, 13, 89) and oat (112, 113). The induction is, however, specific to only some of the several isoenzymes (89, 113, 4). In wheat half-seeds, abscisic acid causes inhibition of GA_3-induced release of the enzyme but has no effect on the GA_3-induced enzyme (5). Low concentration of the drug chloramphenicol also

stimulates the acid phosphatase of germinated cotton embryos (21).

2.2.1 Multiple forms

Several plants contain multiple isoenzymes of acid phosphatase which vary in size and other properties. Multiple forms of the enzyme have been demonstrated in tea leaves (14), tomato fruit (14, 51), cell wall of the cultured cells of rice (116), wheat roots (104),several grass seeds, coleoptiles and leaves (150), yam tubers (121, 122), aleurone particles of rice grains (268), sunflower seeds (192), cotyledons of Vigna mungo (248), cytoplasm and membrane fractions of Phaseolus vulgaris (165), Spirodela oligorrhiza (207) and the plasma membrane of peanut cotyledons (17). Multiple acid phosphatases have been reported in the thylakoid membranes of wheat that are significantly different from the enzymes involved in phosphoprotein phosphorylation (270). Whereas the seed-coat of pea cotyledon contains a tissue specific isoenzyme that has not been detected in any other part of the cotyledon (173), there are conflicting reports on the numbner of isoenzymes in the cotyledons themselves (119, 130, 173). While Murray and Collier (173) have reported the presence of one major and four minor isoenzymes in the cotyledons of developing pea seed, Johnson et al. (119) have observed only three isoenzymes in the cotyledons of germinating peas which differ in their emergence and resistance to freezing. In contrast, Konopska and Sakowski (130) havealso reported the occurrence of three isoenzymes in imbibed

pea aleurone grains but only one of the three isoenzymes persists while the other two disappear with increase in germination period.

2.2.2 Glycoprotein nature of the enzyme

Several plant acid phosphatases have been demonstrated to be glycoproteins by the phenol-sulphuric acid method, binding to concanavalin A and/or staining with Shiff's reagent (62, 81, 86, 88, 116, 121, 122, 150, 192, 193, 235, 267). The violet-coloured acid phosphatase of sweet potato contains 8.8 - 9.8% neutral sugars composed of fucose, xylose, arabinose, mannose and galactose. Only one hexosamine, glucosamine, has ben detected and accounts for about 2.9% by weight (88). The carbohydrate portion of sycamore cell wall enzyme is very small (18 residues) compared to the polypeptide chain (950 amino acid residues) (62). The cytoplasmic and membrane-bound enzymes purified from yam tuber contain almost equal proportions of hexoses (7%) and hexosamines (4%) (122). About 9% of the purified enzyme from rye germ is neutral sugars and 25% of them are pentoses (81). Three neutral sugar values of 6%, 7 - 12% and 16% have been reported for the acid phosphatase isolated from maize scutellum at different stages of germination (218, 250).

It seems that fungal acid phosphatases contain high percentage of carbohydrate than higher plant acid phosphatases. The inducible acid phosphatase from Saccharomyces cerevisiae contains 50% carbohydrate composed of 45% mannose with traces of

glucose and 5% N-acetylglucosamine (162). The purified enzyme from S. cerevisiae culture fluid contains 35 - 40% carbohydrate consisting mainly of mannose and glucosamine (133). 57% of the enzyme from Rhodotorula ruba has been reported to be carbohydrate (275). Candida albicans enzyme is a mannoprotein with hexose and protein in the ratio of 7:1 (184). The i form of acid phosphatase from Aspergillus niger contains 31% carbohydrate consisting of glucosamine, mannose and galactose, the latter two are in the ratio of 89 : 11 (234). In contrast, another variety of Aspergillus niger contains only 9.5% carbohydrate consisting of galactose and mnnnose, 11.8% of this carbohydrate content is hexosamine (101). The carbohydrate content of the secreted enzyme purified from Schizosaccharomyces pombe accounts for 90% of the total molecular mass (230).

2.2.3 Localization of acid phosphatase

Acid phosphatase has been reported to be present in various organelles of plant cell. The existance of the enzyme in the cell wall of higher plants is well documented (9, 61, 62, 104, 122, 165, 242). Histochemically the enzyme has been demonstrated to be localized in the endoplasmic reticulum of barley aleurone cells treated with GA_3 (199). In their study on vacuole formation in the actively growing root meristem of barley, Buvat and Robert (44), have demonstrated that, at the beginning of differentiation, the Golgi apparatus gives rise to vacuoles and tubules that concentrate hydrolases including acid phosphatase. During germination, acid phosphatase has been

observed on the plasmalemma, dictyosomes and reticulum vesicles of Allium cepa radicles, in addition to aleurone grains and new vacuoles (169). At early stages of differentiation of xylum cells of tobacco and peas and as the vessels elaborate cell wall thickenings, acid phosphatase has been found to reside in the cisternae of the ER, the dictyosomes, certain vesicles and on the vacuolar membrane. However, at later stages and due to autolysis of the cytoplasm and organelles as well as hydrolysis of primary cell walls and end plates, the enzyme is found within the vacuoles and throughout the cytoplasm (59). The plasma membranes isolated from corn leaf mesophyll protoplast (194) and corn roots (144), are associated with an acid phosphatase activity, but, it has not been shown to be a separate protein (194). Whole cell extract of Phaseolus vulgaris contains several acid phosphatase isoenzymes that are partially segregated subcellularly in the chloroplast, mitochondria and nucleus (180). The Golgi apparatus of pea stem (195, 206) and sycamore cultured cells (7) contains an acid phosphatase. The enzyme is also distributed throughout the entire cisternae of the Golgi apparatus as well as the ER of plants (167). The enzyme has been reported to be present in the dictyosomes and mitochondria of aleunone layers of barley (89). The enzyme is also preferentially located in the fibriller component, within the nucleolus and around the nucleoler organizing region of Allium cepa anthers (219). Acid phosphatase has been detected in several isolated vacuoles (143, 156,). The majority of ATPase hydrolytic activity of vacuoles isolated from tobacoo suspension cultured cell protoplasts in which 13% of acid

phosphatase is recovered, is due to this non-specific acid phosphatase (161). The vacuoles isolated from red beet contain soluble acid phosphatase and partially membrane-bound ATPase which are distinguished by their susceptibility to inhibition by molybdate (143). In yeast, acid phosphatase has been localized cytochemically in vesicles or small vacuoles, central vacuole, in flat vesicles beneath the plasma membrane, ER, nuclear membrane, Golgi-like structures and on the surface (147, 201, 264). Surprisingly, acid phosphatase activity has been found cytochemically in the intercellular spaces in the roots of Nymphoides peltata (188).

2.2.4 General properties of plant acid phosphatases

Some of the properties of plant acid phosphatases are summarised in Table III. The values of pH optima vary between pH 4.0 and pH 6.0. Values of Km and molecular weights vary quite significantly from source to source. For example Km of sunflower enzymes for pNPP is 0.029 mM and 0.098 mM while that of yam tuber cell wall is 3.1 and 3.4 mM. The molecular weight of plant acid phosphatase and their isoenzymes range from 30 kD to 110 kD. SDS-PAGE data show further that some of the enzymes possess subunit structures (87, 192, 256).

2.2.4.1 Substrate specificity

Plant acid phosphatases regardless of their source hydrolyze a wide range of phosphomonoesters though to variable extents. Whereas most acid phosphatases prefer pNPP as

TABLE III : Properties of acid phosphatases from various plant sources

Enzyme source	pH optimum	Km for pNPP (mM)	Molecular Weight (kD)		References
			SDS	Gel filtration	
Yam tuber cytoplasm	5.0	2.85	98	98	121
Yam tuber cell wall (2 isozymes)	4.5,5.2	3.1,3.4	50,56	55,65	122
Sweet potato tuber	5.8	0.068	55	110	256,257
Extracellular enzyme of tobacco cultured cells	5.8	0.3			181
Cultured tobacco cells	4.7	0.56	76	74	235
Sunflower seed		0.029	56,52	103	192
Maize scutella (4 days)	5.4	0.3	65	65	218
Maize scutella (dormant)	5.2	0.26	65		250
Vigna mungo cotyledons	5.6 all iso enzymes	0.13,0.14, 0.25,0.08	76,36, 71,74		248
Tomato fruit 2 isozymes	5.5	0.83			51
Asciepias curassavica latex	6.0	0.69		27	95

substrate, there are others which prefer other substances such as pheylphosphate for the enzyme from rye germ (81), β-naphthylphosphate for yam tuber cytoplasmicenzyme (120),β-glycerophosphate for the maize scutellum enzyme (218) and bis-p-nitropheylphosphate for the enzyme from cellwallof tobacco cultured cells (242). A 20-fold purified preparation from immature pea seeds is capable of hydrolysing glucose-6-phosphate more than glucose-1-phosphate (255), whereas a 200 - fold purified preparation from pea leaves possesses the ability of hydrolysing nucleotides (85).

Although not all the enzyme preparations used in such experiment were homogenous, the data on substrate specificity may be misleading. Nevertheless the non-specific nature of plant acid phosphatases, in general, is well established (81, 87, 121, 122, 192, 257, 268).

2.2.4.2 Effectors and inhibitors

Plant acid phosphatases are sensitive to heavy metal ions, F^- and polyanions, although they vary in the potency of inhibitions (81, 192, 235, 248, 257). Enzymes that are unaffected by a wide range of inorganic cations also exist (51, 184, 218). Of special interest is the activation of some enzymes by some metal ions. The cytoplasmic and membrane-bound acid phosphatases from yam tubers are stimulated by Cu^{2+} and Mg^{2+} (121, 122). Also the cell wall-associated acid phosphatase and the isoenzyme IIb of rice endosperm are activated by Cu^{2+}, Mg^{2+},

Co^{2+} and Zn^{2+} (126). The effect of EDTA on the enzyme varies from stimulatory to inhibitory to non-effective. While it activates the enzyme from Avena elatiol seeds (278), and pea cotyledons (255), it inhibits the enzymes from yam tuber (121, 122), and is completely insensitive to sweet potato enzyme (257). Citrate stimulates isoenzymes Ia_2 and Ia_3 of Poa pratensis seeds (149) whereas tartarate activates the enzyme from seeds of triticate (52), Partially purified acid phosphatase from spinach leaves and a commercial preparation of potato tuber acid phosphatase are activated by oxidized glutathione and dehydroascorbate (39).

2.2.4.3 Metalloprotein nature

Several plant acid phosphatases have been reported to contain metals in the their molecules. Sweet potato enzyme has been reported to contain Mn, B and Si (256). The amount of Mn accounts for 2g atom/mol in trivalent rather than divalent state (88). Soybean acid phosphatase contains 0.42 ug Mn/mg dry weight of protein (86).All the six isozymes isolated from cell wall of cultured rice cells contain significant amount of Mn with traces of Ca, Mg and silicon (116). All there enzymes are characterized by violet colour but it has not been determined whether this colour is due to manganese ions or some other factors.

2.2.4.4 Temperature optimum and thermal stability

Some acid phosphatases exhibit maximum activity at

elevated temperature. Temperature optima of 50°C and 60 - 70°C have been reported for enzymes from yam tuber (121, 122) and rape seedlings respectively (217). Aspergillus niger enzyme has an optimum temperature of 37°C (101).

Acid phosphatases seem to resist thermal inactivation at elevated temperatures. Saccharomyces cerevisiae acid phosphatase loses 50% of its original activity after 40 min incubation at 50°C (133) whereas that from the yeast Rhodotorula ruba retains its activity for 60 min at 50°C (275). For Aspergillus niger enzyme inactivation starts at 50°C and the enzyme is completely denatured when maintained at 100°C for 30 min (101). Candida albicana enzyme is completely inactivated at 70°C within 12 min (184). The membrane-bound enzyme of cultured tobacco cells is more stable to heat treatment at 55°C for 40 min than the solubilized form. The former retains 95% of its original activity compared to the latter which loses 50 of its activity in the heat treatment (242). All the isozymes of the cell wall of rice plant cultured cell retain most of their activity at 65°C for 10 min except one isoensyme which resists only upto 50°C (116). The enzymes from yam tuber are stable at 50°C for 20 min and above this temperature inactivation rapidly takes place (121, 122). Isozyme IIa from Vigna cotyledons is unstable to thermal inactivation compared to Ia and IIb. Surprisingly the activity of isoenzyme IIb is enhanced upto 155% by heat treatment (248).

2.2.4.5 Purification

The enzyme has recently been purified to apparent homogeneity from a wide variety of tissues such as cotyledons of Vigna mungo (248), tomato (51, 193), maize scutella (218, 250) cultured tobacco cells (235), sweet potato (256), yam tuber (121, 122), sunflower seeds (192), rice cultured cells (116) and bean hypocotyl (165).

The methods employed in their purification are conventional, which include gel filtration, ion-exchange chromatography on DEAE-cellulose, DEAE-Sephadex, CM-cellulose and CM-Sephadex. Affinity chromatography is sometimes included in the purification procedures (81, 192, 193). Cell wall-bound enzymes are eluted by NaCl (61, 62, 104, 116, 122, 242). Triton X - 100 has been used for the solubilization of the enzyme of Phaseolus vulgaris cell wall, mitochondria and microsomes (165). The membrane-bound enzyme has been purified only from a very few plant sources.

2.2.4.6 Physiological roles

In general, the role of acid phosphatases in plants is not clear. It has been assumed that acid phosphatase may be implicated in mobilization of phosphate (173), and nutrient reserves (83), differentiation of plastids (166) and in the onset and development of senescence (66), though the latter is not a general phenomenon (14). The presence of the enzyme in the nucleus of Allium cepa anthers may contribute to the increase in

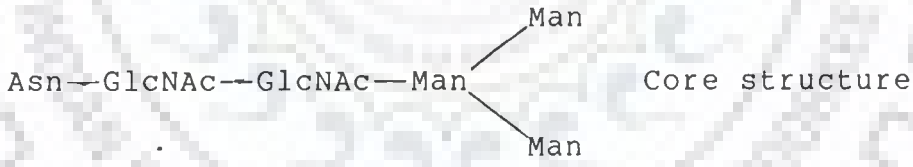
the amount of ortho-phosphate ions and may also participate in the successive trimming of the different pre-ribosomal RNA species that occur during their maturation (219). The lysosomal enzyme of the growing tips of the endophyte of the semiparasitic angiosperm Comandra umbellata is released, probably with other hydrolases, to disrupt the host cell membrane and, therefore, facilitate its invasion (252). Whereas the enzyme in the seed-coat of developing pea cotyledons has been assumed to play considerable role in supplying the developing embryo with inorganic phosphate and other nutrients (173), those in other parts of the cotyledons are of no functional significance (174). Cell wall phosphatases of Acer pseudoplatanus hydrolyze different nucleotides and therefore, participate in regulation of the cell metabolism (155). Besford (20), have suggested that measurement of acid phosphatase in leaves may be a useful test for phosphorus status and an alternative diagonestic procedure to foliar nutrient analysis.

2.3 Glycoproteins and glycoprotein enzymes

Glycoproteins are conjugated proteins which contain covalently linked one or more heterosaccharides of relatively low sugar residues (131). Three types of carbohydrate peptide linkages have been established in plant glycoproteins: N-glycosidic linkage involving C-1 of N-acetyl-D-glucosamine of the oligosaccharide and the amide nitrogen of asparagine in the peptide chain, alkali labile 0-glycosidic linkage involving, D-galactose of the oligosaccharide and serine of the peptide chain,

and the alkali stable 0-glycosidic linkage involving L-arabinose and hydroxyproline or D-galactose and hydroxyproline (231). Glycosylated hydroxyproline seems to be unique to the plant kingdom (131).

The N-glycosidic linkage is found in most living tissues (231) and in membrane glycoproteins in particular (210). Glycoproteins having this kind of linkage are either simple with only D-mannose and N-acetyl-D-glucosamine (the high mannose type) or complex which contain in addition D-galactose, L-fucose, D-xylose and sialic acid (76, 231). The GlcNAc-asparagine linked glycoproteins share a common core of two GlcNAc residues to which is attached the branched mannose trisaccharide (76, 231), and only beyond this point, do the high mannose and the complex types differ.



In fungi (142), and animals (191), the oligosaccharides that are linked N-glycosidically to glycoproteins are assembled from their respective nucleotide sugar precursors on the lipid-intermediate, dolichol phosphate. Oligosaccharides linked 0 - glycosidically to proteins are assembled by direct transfer of the sugars from the sugar nucleotide precursors to the protein acceptor (124). In animals the $Glc_3Man_9GlcNAc_2$ oligosaccharide, is first assembled on the lipid carrier and then transferred en bloc to the polypeptide chain (191).

The peptide chain of glycoproteins whether integral or secretory, are translated on membrane-bound ribosomes (189). The amino acid terminal of the nascent peptide possesses a signal sequence which attaches the ribosomes to a receptor site on the ER (23, 24). This ribosomal binding unit of the ER creates a channel for the passage of nascent polypeptide into the cisternal space of the ER (23, 24). The transfer of $Glc_3Man_9GlcNAc_2$ oligosaccharide to asparagine residue of the peptide chain and the formation of N-glycosidic linkage takes place at the nascent peptide level while the protein is still attached to the RER (127), or after chain completion and release from ribosomes (19). After transfer of the oligosaccharide from the lipid carrier, the oligosaccharide undergoes a number of processing or trimming reactions. All the 3 glucose residues are removed stepwise (254). A glucosidase which removes the glucose residues has been detected in the thyroid ER (237). For some glycoproteins, trimming stops after the removal of the glucose residues to give the high mannose type, while for others the processing continues by the removal of one or more mannose residues to give a family of high mannose oligosaccharides, whereas still for others, 6 mannose residues are removed to give the complex type oligosaccharides (76). α -mannosidase that removes mannose residues, has been isolated from rat liver Golgi apparatus (244). After removal of the glucose residues, the $Man_9GlcNAc_2$-protein is then transported to the Golgi apparatus (216) where a number of mannose residues are removed by the Golgi α -mannosidase. GlcNAc, galactose, sialic acid and probably fucose are then added

stepwise by respective Golgi glycosyltransferases (35, 76, 246).

Higher plant glycoproteins include lectins, reserve polymers, structural proteins, toxins and enzymes. Only few higher plant enzymes such as bromelain, ficin, peroxidaes and invertase have been conclusively proved to be glycoproteins (231), compared to fungi and yeasts in particular. In yeasts, several enzymes have been shown to be glycoproteins. Among these are α-mannosidase, alkaline phosphatase, invertase, asparaginase II, α-galactosidase and β-glucanase (57).

2.4 Intracellular transport

Two models have been proposed to explain the transport events of membrane components and secretory products : the membrane flow (172) and the membrane shuttle (189). In the former model the endomembrane system is viewed as a continuous system with a flow of information through the cells connecting the nuclear membrane, the ER, GA, PM, tonoplast and the external envelopes of chloroplasts and mitochondria. In the membrane shuttle model, each zone of the membrane system is a separate entity and communication between them is through vesicular packages of information.

Proteins have to be transported from the site of synthesis to the location of their function. Thus, the secretory proteins synthesized on the RER have to travel to the periphery of the cell in order to be exported. By studying the secretory process of the mammalian pancreatic exocrine system, it has been shown

that initial precursors of the secretory product appeared first in the RER and then the GA and finally in the zymogen granules (45). This model has since been extended to other systems and it is currently the most accepted scheme (189).

It has been suggested that the carbohydrate moiety of glycoproteins may play a role in their transport (77). Investigations have been focused on the involvement of glycosylation in the insertion of proteins in membranes and their transport to other intracellular membranes and to the cell surface, in viral and animal systems. In such investigations, glycosylation deficient mutants as well as the antibiotic tunicamycin which specifically inhibits glycosylation of N-glycosidically linked glycoproteins (251), are employed.

When core-glycosylation is inhibited by tunicamycin, some glycoproteins are secreted (239), whereas other non-glycosylated forms are not observed at the cell surface (140, 229), due to retention, proteolysis and aggregation within the cell (94, 140, 185). Glycosylation is found not to be essential for the insertion and cleavage of membrane proteins of visicular stomatitis virus (215), and Semliki Forest virus (93). However, the characterization of a specific RER glycoprotein with composition similar to the N-linked oligosaccharides (32), and the proteolytic attack of the non-transported, non-secreted and non-glycosylated forms (185, 140) are not in favour of a possible role of core-glycosylation in intracellular transport.

Late glycosylation has been also shown not to be involved in transport since the intracellular transport is not disturbed when glycoproteins are synthesized in viral cells deficient in glycosyltransferases specifically involved in the processing events in the GA (224).

Not all proteins synthesized on the RER are secreted. Some enzymes are destined to the lysosomes and other organelles. A signal seems to be essential to distinguish between these two types of proteins. It has been postulated that enzymes undergo a secretion-endocytosis process during their way to the lysosomes (106). It has also been found that many molecules enter mammalian cells by a process called receptor-mediated endocytosis (179). Their uptake is governed by recognition receptors at the molecules and cell surface. There are also surface receptors for a variety of glycoprotein enzymes destined for lysosomes (107). The lysosomal enzymes possess a mannose-6-phosphate residue (123) attached to C_6 of mannose of the core region of N-linked oligosaccharides (178, 265) which function as a recognition marker for their transfer to lysosomes. Blockage of glycosylation resultts in increased secretion, loss of lysosomal enzymes and loss of cell surface lysosomal enzymes (178). This shows that N-glycosylation is not essential for the transport of lysosomal enzymes from the RER to PM and that the phosphorylated sugar may serve as a selective segregation signal.

Transport of secretory products in fungi is studied in connection with the mode of deposition of cell wall materials

during budding in yeast. Budding in Saccharomyces cerevisiae starts by the vesiculation of the ER and the transport of the resulting vesicles to the site of bud formation (168). The vesicles contain oxo-β-glucanases and hence are termed glucanases (157). The glucanase vesicles are found to contain acid phosphatase too (147).

A more precise picture is formed when acid phosphatase is used as a secretory marker in protoplasts or yeast whole cells. Histochemically the enzyme is localized in all organelles involved in the process of biosynthesis and secretion of glycoproteins (264). The enzyme has also been reported to occur in vacuoles, Golgi structures, vesicles and cell walls (147). It has also been shown cytochemically to be localized in the central vacuole, surface of the cell envelope, surface of the plasma membrane and within adjacent vesicles apparently part of the ER (201).

The role of asparagine-linked oligosaccharides in the secretion of several fungal enzymes is studied by blockage of glycosylation by the drug tunicamycin. Although the effect of the drug on the biosynthesis and secretion vary widely, it indicates that transport of hydrolases is not affected by the absence of the carbohydrate moiety. Non-glycosylated exo-β-glucanase is transported and secreted and no accumulation of the active molecule is observed in the cytoplasm (232). Further the transport of non-glycosylated external invertase, acid phosphatase and α-galactosidase is not affected although they are not secreted (135, 232).

Analytical investigation of secretory glycoprotein enzymes reveals that they are bound to membranes during their transport. The external invertase of S. cerevisiae which contains 50% carbohydrate and is composed of two subunits (253), is localized outside the plasma membrane. S. cerevisiae which is actively synthesizing invertase also contains a smaller membrane-bound form (10, 213), which is a precursor of the external one (10). Inhibition of the synthesis of the external (135) and the membrane-bound enzymes (10), by tunicamycin indicates that the membrane-bound form also bears the inner core (10). Precipitation by Con A (213) and isopycnic density gradient centrifugation (214) confirms the presence of carbohydrate in the enzyme molecule.

Acid phosphatase is actively secreted by depressed yeast cells (263). It contains equal amounts of carbohydrate and protein (26). The internal glycosylated forms of the enzyme, which appear to be precursors of the exoenzyme, are found either free or bound to membrane in protoplast lysate (27). Whereas the external enzyme contains more carbohydrate than the membrane-bound as shown by isopycnic centrifugation (27, 214), the membrane-bound is heterogeneous as only a small portion reaches the buoyant density of the external one (27). Further, the external and internal soluble enzymes have equal carbohydrate content as shown by isopycnic centrifugation (214).

More recently intracellular maturation and secretion of acid phosphatase of Saccharoymyces cerevisiae is reported. Three

unglycosylated precursors are synthesized into the membrane of the ER where they are core glycosylated in a membrane-bound form. At the Golgi, outer carbohydrate chains are attached to the core and the enzyme appears in a soluble form, indicating release from the membrane between the ER and the GA. The time required for the enzyme synthesis and transport to the GA is about five minutes (225). Glycosylation and secretion of a phosphate repressible acid phosphatase of the fission yeast Schizosaccharomyces pombe has also been recently described. Two intracellular precursors of the enzyme are detected : the unglycosylated form which accumulates in the presence of tunicamycin, and a partially glycosylated form which accumulates mostly in membranes of cells grown in rich medium. In minimal medium these two forms are rapidly processed to the fully glycosylated enzyme. The precursors are not secreted or transported to the PM (230).

Very recently it has been shown that glycosylation is not essential for the transport of phytohemagglutinin in developing Phaseolus vulgaris cotyledons as the unglycosylated form reaches its site of accumulation, the protein bodies (29). Glycans are also not required for the transport of the vacuolar α-mannosidase out of the ER-Golgi system in jack bean cotyledons (80).

Monensis slows down the proteolytic processing (pro-protein to protein) of rice lectin precursor in developing rice embryo to mature rice lectin as well as the glycolytic processing (removal of peripheral N - acetylglucosamine residues) of

phytohemagglutinin in developing bean cotyledons (238). This inhibition of processing has been attributed to the alkalination by monensin, of the compartments in which these proteins accumulate (vaculoes and protein bodies) (238). Monensin further, inhibits the secretion but not the synthesis of α-amylase and acid phosphatase and four other proteins from barley aleurone layers by causing their accumulation within the protoplasts although the effect is unequal among the α-amylase isoenzymes (159). The accumulation of these enzyme activities is localized in an organelle with a buoyant density similar to Golgi apparatus isolated from various plant sources (1.15 - 1.16). Monensin, thus inhibits the secretion of the proteins by influencing their intracellular transport (159).

From the above mentioned literature review, the following conclusions emerged :

(i) Although the plant endomembranes in pure form are difficult to obtain, precise manipulations of gradient, together with the use of marker enzymes would allow preparation of highly enriched fractions of PM, GA and ER free from cross contamination.

(ii) Membrane-bound acid phosphatases have not been purified to homogeneity or studied in greater detail and their physiological role is also not clearly established.

(iii) Most of the acid phosphatases, including those bound to plasma membranes, are glycoproteins and thus may serve as marker enzymes for studying the intracellular transport of proteins in plant seeds.

3.0 EXPERIMENTAL PROCEDURES

3.1 Materials

Pea seeds (Pisum sativum L.) variety Azad (wrinkled variety), were purchased from the local market.

Nucleoside mono, di-, triphosphates, glucose-1-phosphate, glucose-6-phosphate, glyceraldehyde-3-phosphate and inosine diphosphate were purchased from Pierce Chemical Company (U.S.A). Triton X-100, sodium dodecyl sulfate, sodium deoxycholate, sodium taurodeoxycholate, Nonidet NP-40, monensin, UDP-glucose, DEAE-Sephadex, CM-Sephadex, Sephadex G-100, Sephadex G-200, endo-N-acetyl- β-D-glucosaminidase H, blue dextran,, dolichol monophosphate, p-nitrophenyl phosphate and Brij-35 were obtained from Sigma Chemicals Company (St. Louis, U.S.A.). Acrylamide, N, N´,methylene-bis-acrylamide, CHAPS, TEMED and Tris were from Serva (FRG). Protein standards were from Bio-Rad (U.S.A.). Radioactive UDP [^{14}C] glucose (260 Ci/mol) GDP-[^{14}C]-mannose (307 Ci/mol), tritium-labelled sodium borohydride, radioactive labelled mannitol were purchased from the Radiochemical Centre, Amersham (U.K.). All other chemicals were reagent grade obtained from various commercial sources.

3.2 Methods

3.2.1 Germination and imbibition of seeds

Pea seeds were germinated in the dark at 20 - 25^{o}C in a seed germinator. Healthy seeds were surface sterilized with 0.1%

NaOCl for 5 min followed by thorough washing with tap water to remove the disinfectant. They were then spread on 6 layers of moist cheese cloth in a plastic tray, covered with moist cheese cloth and allowed to germinate in a germinator at 20 - 25°C. The seeds were washed twice every day with running tap water to prevent fungal infection. Wherever no germination was required, the seeds were imbibed by immersing them in sufficient water for 18 hours under the same conditions.

3.2.2 Preparation of membrane fractions

3.2.2.1 Preparation of the crude microsomal fraction

Unless otherwise stated, all operations were carried out at 0 - 4°C.

The crude microsomal fraction was prepared by differential centrifugation as described by Bonner (31). The seeds were decoated, the cotyledons excised from the embryonic axis and rinsed thoroughly with chilled water. The cotyledons were suspended in the homogenization buffer (50 mM Tris-HCl pH 7.4, 0.25 M sucrose, 3 mM EDTA and 0.1% 2-mercaptoethanol) in the ratio of 1:2 (w/v). They were homogenized in a Waring blendor by 3 bursts of 30 seconds each. The homogenate was filtered through four layers of cheese cloth. In initial experiments 100 cotyledons (ca 20 g) of germinated seeds at various stages of development were used. Routinely 1000 cotyledons (ca 200 g) of 18 h imbibed seeds were homogenized in two batches. The filtrate was then centrifuged at 12,000 x g for 20 min in Beckman J2-21

centrifuge using JA-20 rotor. The resulting supernatant was further centrifuged at 105,000 x g for 60 min in Centrikon T-2060 centrifuge using Centrikon TFT 70-38 rotor. The clear supernatant (cytosol) was decanted and retained for some preliminary assays. The fluffy material which remained unsettled was separated from the pellet and saved for further analysis.

The pellet was resuspended with the help of a glass rod with smooth end and by passing through a narrow 2 ml pipette in 4 ml of suspension buffer (50 mM Tris-HCl pH 7.4, 0.25 M sucrose and 0.1% 2-mercaptoethanol) and used in some preliminary experiments.

3.2.2.2 Isolation of the plasma membrane

The plasma membrane-rich fraction was prepared by the method of Dupont *et al.* (73) with minor modifications as described by Sharma *et al.* (233). The microsomal fraction consisting mainly of plasma membrane, Golgi apparatus and endoplasmic reticulum, prepared as described above, was suspended in 30 ml of gradient buffer (10 mM Tris-HCl buffer pH 7.4) containing 20% (w/w) sucrose and 1 mM 2-mercaptoethanol. Ten ml of this suspension were layered carefully over 15 ml of 34% (w/w) sucrose in the gradient buffer. The fluffy material was made to 20% w/w sucrose and 10 ml of this were also layered over 15 ml of 34% w/w sucrose in the same buffer and the samples were centrifuged at 105,000 xg for 1.5 h. The clear sucrose layers were carefully removed by Pasteur pipette. The membranes banding

at the interface of 20% / 34% sucrose were removed and saved for the preparations of Golgi apparatus and ER in subsequent steps. The 34% (w/w) sucrose and fluffy material below the interface were decanted and the pellet containing the plasma membrane was suspended in 4 ml suspension buffer.

The plasma membrane (pellet) was further purified by a discontinuous sucrose density gradient centrifugation method as described by Hall (102). Two ml of the PM pellet resulting from the previous step (ca 15 mg), were layered on a six-step sucrose gradient of 4 ml of 45% and 6.4 ml of each of 38, 34, 30, 25 and 20% sucrose in gradient buffer in 38 ml tubes. The tubes were centrifuged at 95,000 x g for 2 h in Centrikon TFT 70.38 rotor. The plasma membrane banding at the interface of 45%/38% corresponding to a density of 1.17 g/cm^3 was identified by the presence of its marker enzyme, glucan synthase II.

3.2.2.3 Preparation of Golgi enriched membrane fraction

The membrane fraction banding at the interface of 20%/34% (w/w) sucrose, obtained from the above step, was used for the preparation of Golgi apparatus and endoplasmic reticulum by sucrose density gradient method of Green (99) with some minor modifications. The 20/34% sucrose interface membrane fraction, containing largely Golgi apparatus and endoplasmic reticulum membranes, was diluted to about 10% sucrose by suspension buffer without sucrose and pelleted by centrifugation at 105,000 xg for 60 min. The pellet was resuspended in 4 ml of gradient buffer

containing 20% (w/w) sucrose and layered carefully on a 3-step sucrose density gradient system composed of 7 ml of each of 43%, 37% and 25% (w/w) sucrose successively in the same buffer in 25 ml tubes and centrifuged at 105,000 x g for 3 h in Centrikon T-2060 centrifuge and TFT 70 - 38 Centrikon rotor. A major band at the interface of 37%/25% (w/w) sucrose was formed. The membrane suspension above the 37%/25% interface band was carefully removed by Pasteur pipette and retained for the preparation of the endoplasmic reticulum. The membrane fraction banding at the of 37/25% corresponding to a density of 1.15 g/cm^3 and representing enriched Golgi apparatus was recovered and diluted to 10% sucrose by suspension buffer without sucrose and pelleted by centrifugation at 105,000 xg for 60 min. The resulting pellet was then suspended in 2 ml suspension buffer and used in all experiments concerning Golgi apparatus.

3.2.2.4 Preparation of endoplasmic reticulum

The membrane suspension staying above the 37/25% interface band was diluted to 10% sucrose with suspension buffer without sucrose and centrifuged at 105,000 x g for 60 min as before. The resulting pellet was resuspended in 2 ml suspension buffer and used in all experiments concerning the endoplasmic reticulum.

3.2.3 Solubilization of the membrane-bound enzyme

Plasma membrane fraction (20 mg/ml) was suspended in Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 5 mM EDTA and 0.1% 2-mercaptoethanol. To 2 ml membrane suspension, 4 ml of 1.5%

(w/v) CHAPS solution in the same buffer were added dropwise with gentle stirring on a Vortex cyclomixer for 1 x 4 min so that the final detergent concentration and the protein to detergent ratio were 1% and 2:3 respectively. After 30 min incubation in ice, the homogenate was centrifuged at 105,000 x g in Beckman type 50 Ti rotor for 60 min. The clear supernatant was carefully separated from the pellet with the help of a Pasteur pipette and used as solubilized enzyme. The pellet obtained at 105,000 x g was resuspended in the suspension buffer with a Teflon tissue homogenizer and assayed for the enzyme activity that remained unsolubilized by the detergent used. The percent solubilized activity was calculated assuming the sum of the activities in the supernatant and suspended pellet as 100 percent. Solubilization of the acid phosphatase bound to GA was carried out exactly under the same conditions.

3.2.4 DEAE-Sephadex column chromatography

The fresh ion-exchanger was suspended in distilled water overnight. The water was decanted and the resin was suspended in HCl solution (0.5 N) for 30 min. After sedimentation, the acid was decanted and the sorbent was next suspended in water, transferred to a sintered glass funnel and thoroughly washed with water to remove the acid. The washed sorbent was then suspended in 0.5M NaOH containing 0.5M NaCl for 30 min and washed as above. The washed resin was finally suspended in water containing 0.25 M NaCl and stored until its further use when it was washed thoroughly with water and then equilibrated with the

equilibration buffer to be used in the chromatography and packed in the column.

The solubilized enzyme from PM (ca 26 mg protein) was dialyzed overnight against 1 litre 10 mM Tris-HCl buffer, pH 7.4, containing 1mM 2-mercaptoethanol. The dialyzed enzyme was applied on the pre-chilld DEAE-Sephadex A 50 column (1.0 x 12 cm) pre-equilibrated with the same buffer. The column was washed with 25 ml equilibrating buffer to remove the unadsorbed proteins.

The adsorbed proteins were eluted by a linear gradient from 0 to 250 mM NaCl using a single mixing container with 50 ml 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM 2-mercaptoethanol and a reservoir with 50 ml of the same buffer containing 250 mM NaCl. Fractions (1.5 ml) were collected at a flow rate of 15 ml/h. Aliquots (0.1 ml) from every other fraction were assayed for protein content and acid phosphatase activity. The peak enzyme containing fractions were pooled and used for CM-Sephadex column chromatography.

3.2.5 CM-Sephadex column chromatography

The ion-exchanger was charged in the same manner as DEAE-Sephadex except that the alkali was used first and the acid was used last. The enzyme fractions eluted from DEAE-Sephadex (peak II, PM-APase II; 3.2 mg protein) were pooled and dialyzed overnight against two changes of 1 litre 25 mM sodium acetate buffer, pH 5.0, containing 1 mM 2-mercaptoethanol and cleared by

centrifugation at 10,000 xg for 5 min. The clear supernatant (2.1 mg protein) was applied to CM-Sephadex column (1.0 x 5 cm) pre-equilibrated with the same buffer. The column was washed with 15 ml equilibrating buffer to remove unadsorbed protein. The adsorbed proteins were eluted by a linear NaCl gradient (0 - 500 mM) using a single mixing container containing 50 ml of equlibrating buffer and a reservoir with 50 ml of the same buffer containing 500 mM NaCl. Fractions (1.5 ml) were collected at a flow rate of 15 ml/h. Aliquots (0.2 ml) from every other fraction were assayed for protein and acid phosphatase. Top active fractions were pooled, dialyzed, concentrated under nitrogen and used for electrophoresis. Pooled active fractions from Sephadex G-100 gel filtration column containing GA-APase II were chromatographed in the same manner.

3.2.6 Gel filtration on Sephadex G-100

Sephadex G-100 was suspended in 25 mM sodium acetate buffer, pH 5.0, containing 1 mM 2-mercaptoethanol and allowed to swell for 4 to 5 days at room temperature (25^{o}C). The floating fine particles were removed by repeated decantations. The Sephadex slurry was then deaerated under reduced pressure with the help of a water suction pump and finally packed under gravity in a double-walled glass jacketed column (1.5 x 50 cm) fitted with glass wool at the bottom. The column was fully equilibrated with the same buffer and its temperature was maintained at 4^{o}C $\pm$ 2 by circulating ice cold water in outer jacket.

The solubilized GA-APase (11.4 mg) was dialyzed overnight against the equilibrating buffer. Precipitated proteins were removed by centrifugation at 10,000 xg for 5 min and the clear supernatant (6.5 mg protein) was concentrated to 2 ml by lyophilization and loaded on the pre-equilibrated cold column. The proteins were eluted with the same buffer at a flow rate of 6 ml/h and 3.5 ml fractions were collected. Aliquots (0.2 ml) from every other fraction were assayed for protein and APase activity.

3.2.7 Molecular weight determination by gel filtration on Sephadex G-200

Sephadex G-200 was hydrated and the column (1.5 x 65 cm) was packed and equlibrated in the same manner as was used for Sephadex G-100 column. The standard proteins used were : phosphorylase b (97.4 kD), bovine serum albumin (66 kD), ovalbumin (42.7 kD) and cytochrome c (12.2 kD). The marker proteins (5 mg each) were dissolved in the same buffer, and applied to the column. The proteins were eluted with the same buffer at a flow rate of 6 ml/h. Two ml fractions were collected and assayed for protein. A standard curve was constructed from the elution volumes of the marker proteins and void volume taken as elution volume for blue dextran (M.W. 2 x 10^6). Elution experiments with PM-APase and GA-APase were performed using the same column under identical conditions. The elution volume of the A-Pase was measured by assaying acid phosphatase and protein. The molecular weights of the PM-APase II and GA-APase II were estimated by referring to the standrad curve.

3.2.8 Bio-Gel P-4 column chromatography

Dry Bio-Gel P-4 was suspended in excess 10 mM sodium acetate buffer, pH 5.0, and allowed to hydrate for 4h at room temperature (25^{o}C). After swelling up, half the supernatant buffer was decanted and the slurry was deaerated under reduced pressure with the help of a water suction pump. The column was packed under gravity and equilibrated with the same buffer.

The reduced ^{3}H-labelled sugar alcohols were applied to the column and eluted with 10 mM sodium acetate buffer, pH 5.0. Fractions (1 ml) were collected at a flow rate of 6 ml/h and counted in Beckman liquid scintillation counter. Radioactive labelled mannitol and other oligosaccharides were used as standards.

3.2.9 Enzyme assays

3.2.9.1 Acid phosphatase

Acid phosphatase activity was measured by a slightly modified method of Odds and Hierholzer (184), using p-nitrophenyl phosphate as substrate. The incubation mixture (1.0 ml) contained 10 - 200 µg protein, 1 mM p-nitrophenyl phosphate and 50 mM acetate buffer, pH 5.0. The reaction was terminated after 15 min incubation at 30^{o}C by the addition of 1.5 ml of 4% Na_2CO_3. The absorbance of yellow colour of p-nitrophenol released was measured at 420 nm against the control to which the enzyme had been added after terminating the reaction. Specific activity was

expressed as u moles p-nitrophenol produced per minute per mg protein. When substrates other than p-nitrophenyl phosphate were used, the enzyme activity was assayed by measuring the amount of Pi released. The standard reaction mixture contained 1.0 mM substrate, 50 mM acetate buffer, pH 5.0, and the enzyme preparation (10 - 200 µg protein) in a total volume of 1.5 ml. Incubation was carried out at 30°C for 30 min and the reaction was terminated by adding 0.5 ml cold 20% trichloroacetic acid. Denatured protein (if any) was removed by centrifugation at 5000 x g for 10 min and Pi was determined in the supernatant by the procedure of Fiske and SubbaRow (82). The specific activity corresponds to the u moles of Pi liberated per min per mg protein under the assay conditions.

3.2.9.2 Glucan synthase II (a marker enzyme for PM)

Glucan synthase II activity was determined as described by Ray (205) in the presence of high concentration of UDP-glucose and absence of Mg^{2+}. The membrane fraction (100 µg protein) was incubated at 25°C for 20 min in 0.1 ml reaction mixture consisting of 50 mM Tris-HCl buffer, pH 7.4, 0.1 µCi of UDP-[^{14}C] glucose (260 Ci/mol) and 0.5 mM unlabelled UDP-glucose. The reaction was stopped by the addition of 1 ml of 70% (v/v) ethanol, 50 µl of 50 mM $MgCl_2$ and 50 µl of boiled membrane fraction (200 to 300 µg protein) to improve the recovery of labelled products. The mixture was then immediately boiled for 1 min, allowed to stand overnight at 4°C and centrifuged at 3000xg for 5 min. The precipitated particulate material was washed four

times with 70% ethanol to remove all unreacted radioactive substrate and ethanol soluble by-products. The washed precipitate was suspended in scintillation fluid (dioxane cocktail containing 10% naphthalene and 0.5% PPO in dioxane) and the radioactivity was measured in a Beckman LS1801 liquid scintillation counter.

3.2.9.3 Glucan synthase I (a marker enzyme for GA)

Glucan synthase I was determined as described by Green (99), using low concentration of UDP-glucose and 10 mM $MgCl_2$. Membrane fraction (100 µg protein) was suspended in 0.10 ml of reaction mixture containing 100 mM Tris-HCl buffer, pH 7.4, 20 mM $MgCl_2$, 10 mM cellobiose, 4 mM EDTA, 2 mM 2-mercaptoethanol and 0.1 µCi of UDP-[^{14}C]-glucose (6 nmol). After incubation at 25^{o}C for 90 min, the reaction was stopped by heating the reaction mixture to 95^{o}C followed by the addition of 10 mg powdered cellulose to carry the newly synthesized β-glucans. The precipitate was collected by centrifugation and the unreacted radioactive material was extracted 3 x with 1 ml hot distilled water followed by extraction 1 x with chloroform methanol (1:2 v/v) and 2 x with 1 N NaOH. The alkali insoluble residual pellet was rinsed with 1 ml distilled water and its radioactivity was measured as mentioned above for glucan synthase II.

3.2.9.4 Inosine diphosphatase (IDPase) (a marker enzyme for GA)

Latent inosine diphosphatase was determined as described by Green (99), with minor modifications. Membrane fractions were

stored at 4°C for three days. The membrane fraction (0.1 ml) was added to the substrate solution (0.9 ml) consisting of 3 mM inosine diphosphate (IDP, sodium salt), 1.0 mM $MgCl_2$ and 50 mM Tris-HCl buffer, pH 7.5. After incubation at 25°C for 60 min, the reaction was terminated by the addition of 1 ml cold 10% trichloroacetic acid. Denatured protein were removed by centrifugation at 5000 xg for 10 min and the inorganic phosphate released was determined in the supernatant by the method of Fiske and SubbaRow (82). Two solutions, one without enzyme and the other without IDP, were used as controls.

3.2.9.5 Mannosyltransferase (a marker enzyme for ER)

The transfer of mannosyl from GDP-[^{14}C]-mannose to endogenous as well as exogenous lipid acceptor (dolichol phosphate) was measured by the method of Lehle et al. (141). The reaction mixture consisted of 50 mM Tris-HCl, pH 7.4, 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 0.1 μCi GDP-[^{14}C]-mannose. The particulate membrane fraction (100-200 μg protein) was incubated with the reaction mixture in a final volume of 70 μl. The incorporation of mannose to the exogenous lipid carrier was determined by mixing 10 μl of dolichol monophosphate (10 μg) with 10 μl of 0.1 M Mg-EDTA and dried under nitrogen. The dried lipid was dispersed with 10 μl of 5% Nonidet and incubated with the reaction mixture as above. After 30 min incubation, the reactions were terminated by the addition of 2 ml of chloroform/methanol (3:2, v/v). The precipitated protein was separated by centrifugation and the soluble portion was partitioned with 0.4 ml of 4 mM $MgCl_2$

solution. After thorough mixing, the phases were separated by centrifugation. The upper aqueous phase was discarded and the lipid phase was washed by the method of Folch et al. (84). The washed lipid was dried in a vial and the radioactivity was measured after suspending the dried material in scintillation fluid (dioxane cocktail). Blank was prepared under the same conditions and the enzyme was added after terminating the reaction.

3.2.0 Protein estimation

Protein was estimated by the method of Lowry et al. (154) using bovine serum albumin as a standard. However before carrying out Lowry method, 2-mercaptoethanol was removed by heating the protein samples at 60°C for 30 - 60 min in a water bath (249).

3.2.11 Estimation of total carbohydrate content

Total carbohydrate content was determined by the phenol/sulphuric acid method (71). An appropriate volume of the enzyme (20 - 50 µg carbohydrate) was diluted with distilled water to a final volume of 2 ml. Then 50 µl of 90% phenol solution and 5 ml of concentrated sulphuric acid were added followed by thorough mixing on a Vortex mixer. The mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 485 nm against a blank to which no sample was added. The amount of carbohydrate was computed from the standard curve of glucose prepared under identical conditions.

Carbohydrates were located on gels according to Zacharius et al. (279).

3.2.12 Cleavage of N-glycosidic linkage by endo-N-acetyl-β-D-glucosaminidase H

The glycoprotein enzyme (200 - 500 µg) was incubated with 50 mU/ml endo-N-acetyl- β-D-glucosaminidase H (endo-H) in 50 mM sodium citrate buffer, pH 5.0, at 37°C for 20 h. The reaction was terminated by the addition of 3 volumes of ice cold ethanol and the denatured proteins were precipitated by centrifugation at 10,000 xg for 15 min. The supernatant was decanted and retained and the precipitate was washed twice with 1 ml 75% ethanol and recentrifuged. The supernatant and washings were pooled. The carbohydrate content of both the precipitate and supernatant were determined by the phenol-sulphuric acid (71). The decrease in the carbohydrate content of the acid precipitated protein and the corresponding increase in the carbohydrate content of the supernatant indicated the removal of the carbo-hydrate by the enzyme.

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

SDS-PAGE was carried out by the procedure described by Laemmli (137). The various solutions used were as following:

Stock solutions:

Solution A : 30% acrylamide (w):

Prepared by dissolving 29.2 g acrylamide and 0.8 g bis in

water and made to 100 ml with water.

Solution B : 1.5 M Tris-HCl buffer, pH 8.8:

Prepared by dissolving 18.17 g Tris and 0.4 g SDS in water and the pH was adjusted with HCl and made up to 100 ml with water.

Solution C : 0.5 M Tris-HCl buffer, pH 6.8:

Prepared by dissolving 6.06 g of Tris and 0.4 g SDS in water and the pH was adjusted with HCl and made up to 100 ml with water.

Solution D : 10 ammonium persulphate:

Prepared just prior to use by dissolving 0.1 g ammonium persulphate in 1 ml water.

SDS stock solution : 10% (w/v)

Electrophoresis buffer : Tris-glycine, pH 8.3

It contained in final concentration, 0.025 M Tris, 0.192 M glycine and 0.1% SDS. It was prepared by adding 10 m of 10% SDS stock solution to 3 g of Tris and 14.4 g glycine and made to 1000 ml with water.

Sample buffer : Tris-HCl, pH 6.8

The samples contained in final concentration 0.0625 M Tris-HCl buffer, pH 6.8, 2% SDS, 10% glycerol and 5% 2 - mercaptoethanol.

Marker dye :

1 mg bromophenol blue

100 µl glycerol

900 µl water

Staining solution :

0.1% coomassie brilliant blue R - 250

25% methanol

10% acetic acid

water

Mixed in the ratio of v/v/v and filtered before use.

Working solution

Separating gel :

The separating gel (7 cm) of 10% acrylamide was prepared so that the final concentration of the buffer ws 0.375 M Tris - HCl, pH 8.8, and SDS 0.1%. Polymerization was affected chemically by the addition of 0.025% TEMED and ammonium persulphate and prepared as following :

Solution A	6.0 ml
Solution B	4.5 ml
Solution D	30 µl
TEMED	5 µl

Water upto 20 ml.

Stacking gel:

The stacking gel (1 cm) of 4.5% acrylamide was prepared so that the final concentration was 0.125 M Tris-HCl, pH 6.8 and

0.1% SDS and polymerized as in separating gel and prepared as below :

Solution A	0.9 ml
Solution B	1.5 ml
Solution D	15 µl
TEMED	5 µl

made upto 6 ml with water.

The electrophoresis buffer was applied to the lower chamber and the gels were fitted in their places in the apparatus. Sample proteins were concentrated, mixed with the sample buffer and heated by immersing them in boiling water for 2 min to effect dissociation. Protein standards were treated in same manner. Protein samples were layered on the gels, the tracking dye was added, and the gel tubes were carefully filled with the electrophoresis buffer. The upper chamber was finally filled with the electrophoresis buffer. Electrophoresis was carried out with a current of 3 mA per gel tube at 25°C until the bromophenol blue tracking dye reached the bottom of the gel (ca 7h). The proteins were either fixed in the gels with 50% trichloroacetic acid overnight and then stained for 2 h, or directly stained for 4 - 6 h. Destaining was affected by 7% acetic acid. Mobilities were determined relative to the tracking dye.

Native PAGE was carried out in the same manner but without SDS, at 0 - 4°C under nondenaturation conditions.

3.2.14 Preparation of radioactive labelled oligosaccharide standards

The oligosaccharide standards were prepared as described by Badet and Jeanloz (11). ^{14}C-labelled lipid-linked oligosaccharide standards were obtained from calf pancreas microsomes (10 mg protein) and incubated for 30 min at 30°C in 1 ml of 40 mM Tris-maleate buffer, 10 mM $MnCl_2$ and containing 5µCi GDP-[^{14}C]-mannose at pH 6.3. The reaction was stopped with chloroform : methanol (2:1 v/v). The pellet obtained after centrifugation was extracted 3 x with chloroform : methanol (2:1 v/v) to remove small lipid-linked oligosaccharides. The pellet was washed 4 x with 50 percent methanol and then extracted with 10 ml chloroform : methanol : water (10:10:3 v/v/v). The 10:10:3 extract contained lipid-linked oligosaccharides. Oligosaccharides were released by mild acid hydrolysis. A sample was dried under N_2, dissolved in 0.02 N HCl containing 30 percent isopropanol and heated at 100°C for 20 min. The hydrolysate was dried under N_2, neutralized with 0.02N NaOH and desalted on 1 ml column of AG1x8 anion-exchange resin (acetate form) overlaid with AG50Wx8 cation-exchange resin (H^+; 1 ml).

4.0 RESULTS

4.1 Purification of various endomembranes from pea cotyledons

The endomembrane fractions were prepared by combination of differential centrifugation and sucrose density gradient centrifugation techniques outlined in the flow diagram (Fig 4.1) and schematic representation (Fig 4.2).

EDTA was included in the homogenization medium to detach the ribosomes from the rough endoplasmic reticulum membranes. This was essential to introduce density variation between the rough endoplasmic reticulum (RER) and the plasma membrane (PM), since both have similar densities. The smooth endoplasmic reticulum (SER) (without ribosomes) will have lower density than the PM and, therefore, can be separated easily. Since the homogenization was carried out in an isotonic solution, the integrity of plant membranes was largely preserved. The initial centrifugation (12,000 xg for 20 min) was essential to remove the heavy organelles such as nuclei, plastids, mitochondria, the cell wall and other microbodies. The microsomal fraction containing mainly the PM, SER and Golgi apparatus was separated from the cytosol by centrifuging the supernatant at 105,000 xg for 60 min. The PM was separated from the GA and ER by the 2-step sucrose density gradient of 34/20% sucrose. After centrifugation for 90 min at 80,000 x g, the PM settled down in the form of a pellet in the centrifuge tube whereas the GA and ER formed a discrete band at the 34/20% interface. Further purification of the PM was achieved by using the 6-step sucrose density gradient of 45, 38, 34, 30, 25 and 20% sucrose.

FIG 4.1 : Flow diagram for the isolation and purification of different membrane fractions from Pisum sativum cotyledons

Unless otherwise indicated, all steps were carried out at $4^{o}C$.

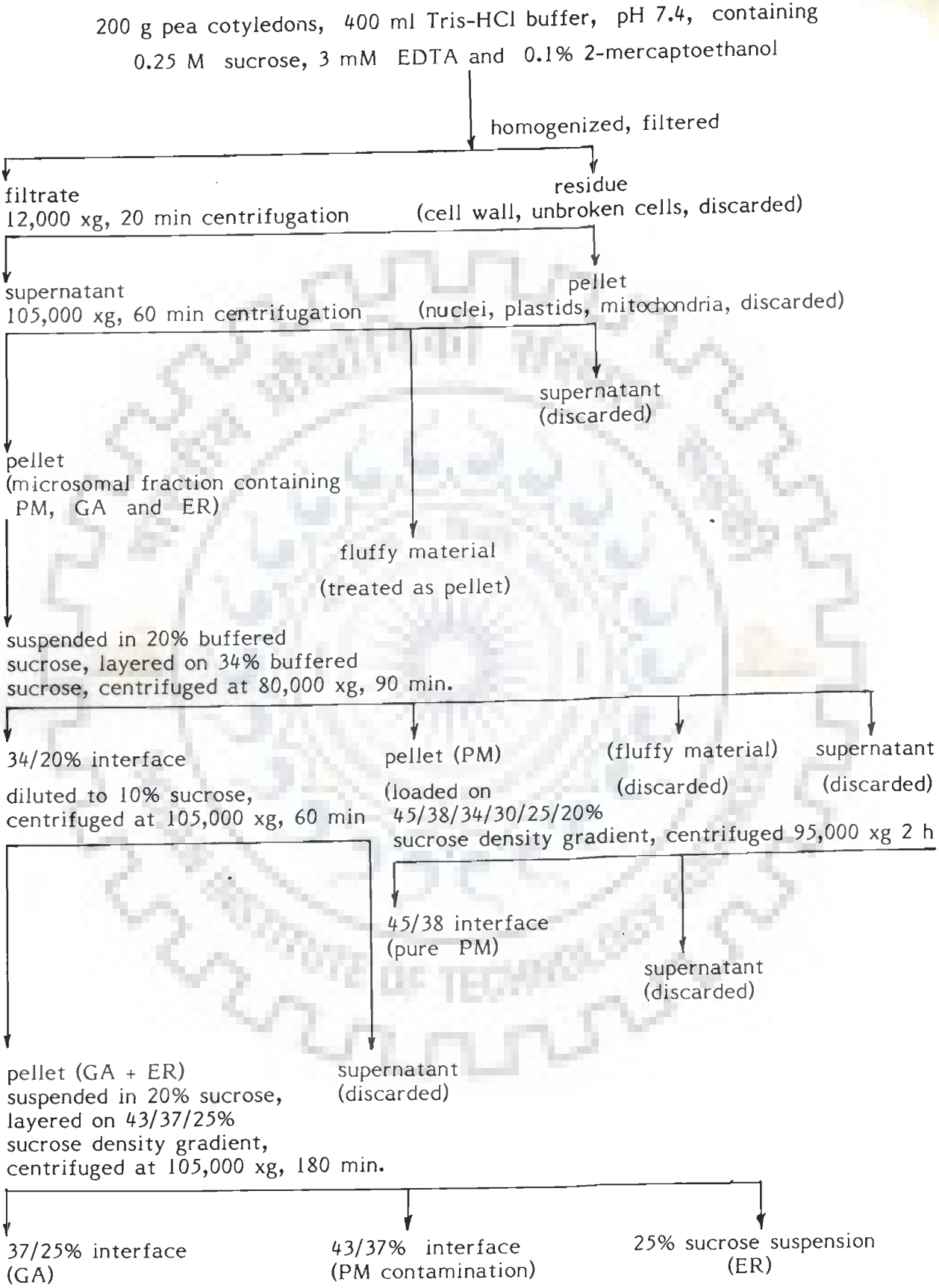

200 g pea cotyledons, 400 ml Tris-HCl buffer, pH 7.4, containing
0.25 M sucrose, 3 mM EDTA and 0.1% 2-mercaptoethanol
homogenized, filtered
filtrate
12,000 xg, 20 min centrifugation
residue
(cell wall, unbroken cells, discarded)
supernatant
105,000 xg, 60 min centrifugation
pellet
(nuclei, plastids, mitochondria, discarded)
supernatant
(discarded)
pellet
(microsomal fraction containing
PM, GA and ER)
fluffy material
(treated as pellet)
suspended in 20% buffered
sucrose, layered on 34% buffered
sucrose, centrifuged at 80,000 xg, 90 min.
34/20% interface
diluted to 10% sucrose,
centrifuged at 105,000 xg, 60 min
pellet (PM)
(loaded on
45/38/34/30/25/20%
sucrose density gradient, centrifuged 95,000 xg 2 h
(fluffy material)
(discarded)
supernatant
(discarded)
45/38 interface
(pure PM)
supernatant
(discarded)
pellet (GA + ER)
suspended in 20% sucrose,
layered on 43/37/25%
sucrose density gradient,
centrifuged at 105,000 xg, 180 min.
supernatant
(discarded)
37/25% interface
(GA)
43/37% interface
(PM contamination)
25% sucrose suspension
(ER)

FIG 4.2 : Separation of various endomembrane fractions by discontinuous sucrose density gradient centrifugation

Step I : Layering of crude microsomes in 20% sucrose on 34% sucrose buffered solution.

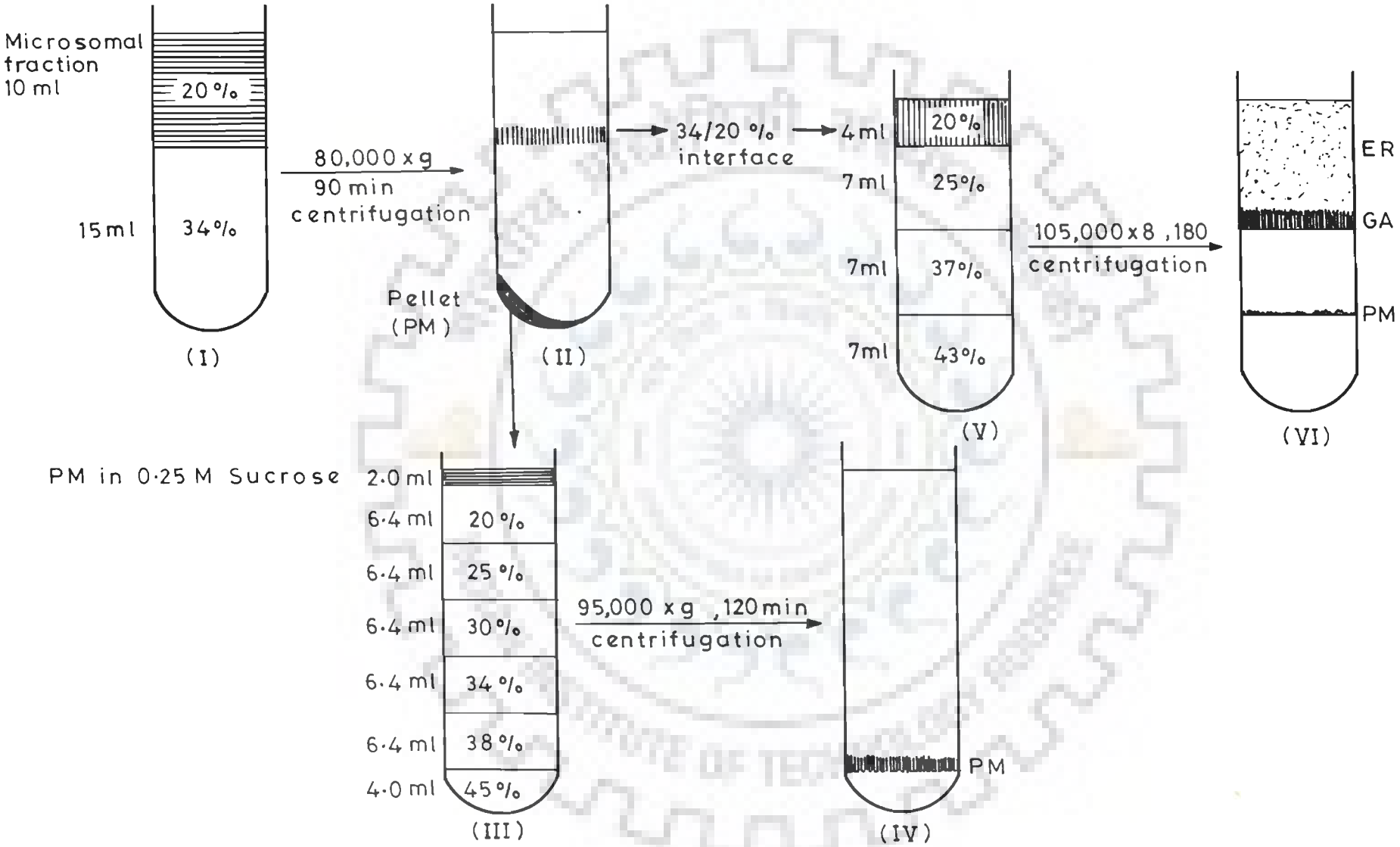
Step II : Separation of PM from GA and ER fractions after centrifugation at 80,000 xg for 90 min.

Step III : Layering of PM pellet from step II in 0.25M sucrose on discontinuous gradient of 45/38/34/30/25/20% sucrose.

Step IV : Purified plasma membrane after centrifugation on the above gradient for 120 min at 95,000 xg.

Step V : Layering of 34/20% interface membrane fraction in 20% sucrose on 43/37/25% sucrose gradient system.

Step VI : Separation of Golgi apparatus fraction (37/25% interface) from the endoplasmic reticulum (suspension above the 37/25% interface) after centrifugation at 105,000xg for 180 min.


Microsomal fraction 10 ml
20 %
15 ml
34 %
(I)
80,000 x g
90 min
centrifugation
Pellet (PM)
(II)
34/20 % interface
4 ml
20 %
7 ml
25 %
7 ml
37 %
7 ml
43 %
(V)
105,000 x 8 ,180
centrifugation
ER
GA
PM
(VI)
PM in 0·25 M Sucrose
2·0 ml
6·4 ml
20 %
6·4 ml
25 %
6·4 ml
30 %
6·4 ml
34 %
6·4 ml
38 %
4·0 ml
45 %
(III)
95,000 x g ,120 min
centrifugation
PM
(IV)

The distribution of the marker enzymes for plasma membrane (PM), Golgi apparatus (GA) and endoplasmic reticulum (ER) was used to identify and to ascertain the purity of the membrane fractions. Our results that are average of three independent experiments are summarized in Table IV. 1,3- β -glucan synthase (glucan synthase II, GS-II), a plasma membrane marker enzyme, was predominantly localized in the membrane fraction banding at 45/38% interface in the 45, 38, 34, 30, 25 and 20% sucrose discontinuous gradients corresponding to an equilibrium density in sucrose of 1.16 - 1.17 g/cm^3. This membrane fraction did not exhibit any significant activity of either inosine diphosphatase, (IDPase) and glucan synthase I (GS-I), marker enzymes for GA, or mannosyltransferase (MTase), the marker enzyme for ER, indicating that the membrane fraction banding at 45/38% interface in the above mentioned discontinuous sucrose gradient was mainly composed of the PM. These results further indicate that the PM was free from GA and ER contamination. On the basis of the specific activity of the PM marker enzyme (GS-II), the PM fraction was purified 12.8 folds over the crude microsomes (12,000-105,000 xg pellet, Table IV). In all experiments, unless noted otherwise, this membrane fraction was referred to as PM and used for the solubilization and purification of PM acid phosphatase (PM-APase).

The band at the interface of 34/20% sucrose gradient containing GA and ER was removed carefully with the help of Pasteur pipette, diluted with 50 mM Tris-HCl buffer, PH 7.4, to

TABLE IV : Distribution of marker enzymes in various membrane fractions of pea cotyledons

Two hundred g of pea cotyledons were used for preparing various membrane fractions. The marker enzymes used to identify them were : glucan synthase II (GS-II) for PM, glucan synthase I (GS-I) and insoine diphosphatase (IDPase) for GA and mannosyltransferase (MTase) for ER. The enzymes were assayed as described under `Methods´. Values are average of three separate preparations.

Fraction	Activities of marker enzymes			
	GS-II(cpm/ mg protein)	IDPase(units/ mg protein)	GS-I (cpm/ mg protein)	MTase[a](cpm/ mg protein)
Crude microsomes(12,000-105,000 xg pellet)	20620±2600	14.8 ± 3.6	2037 ± 260	38024±5000
Plasma membrane 45-38 interface (density 1.17 g/cm^3)[b]	256690±3500	6.5 ± 1.5	1280 ± 220	6312 ± 300 (2883±250)
Golgi apparatus 37-25% interface (density 1.15 g/cm^3)[b]	1066 ± 320	100.3 ± 9	14362 ± 380	6693 ± 496 (3357±360)
Smooth endoplasmic reticulum (density 1.10 g/cm^3)[b]	997 ± 280	7.9 ± 2.5	1302 ± 220	296570±6000 (132355±4000)

a values in paranthesis were obtained without dolichol monophosphate as exogenous lipid acceptor of mannose from GDP [^{14}C] mannose used as the donor substrate.

b Represent the sucrose gradient density at which the bands of membrane fractions are formed.

adjust the sucrose concentration to 10% and pelleted by centrifugation at 105,000 xg for 60 min. The pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.4, containing 20% sucrose and 1 mM 2-mercaptoethanol and then separated by a step gradient consisting of 43, 37 and 25% sucrose solution successively in the same buffer. On centrifugation for 3 h at 105,000 xg, a major band was formed at the 37/25% sucrose gradient interface. The band corresponded to a density of 1.15 g/cm^3. The inosine diphosphatase (IDPase) and glucan synthase I (GS-I), marker enzymes for GA, were exclusively localized in this fraction, while the activities of marker enzymes for PM (GS-II) and ER (MTase) were not significant indicating that the membranes in the 37/25% sucrose gradient interface contained largely GA fraction and were practically free from the cross contamination of PM and ER (Table IV). As judged by the specific activity of the marker enzyme, the GA fraction was enriched by about 7-folds. In all our experiments this fraction was referred to as GA membrane fraction and was used to solubilize the APase.

The suspension above the 37/25% sucrose gradient interface corresponding to a density of 1.1 g/cm^3 was found to be highly rich in the dolichol monophosphate: GDP-mannose mannosyltransferase (MTase) with almost no or very little activity of GS-II and IDPase/GS-I, the marker enzymes for PM and GA, respectively. The membrane fraction obtained from the suspension above the 37/25% sucrose gradient was therefore referred to as ER. On the basis of the specific activity of the

marker enzyme, MTase, about 8-fold enrichment of the ER fraction, relative to the crude microsomes, was achieved.

4.2 Distribution of APase in various subcellular fractions of 18 h imbibed pea cotyledons

Table V shows the distribution of APase activity in the particulate and cytosol fractions. The 12,000 x g supernatant, which is essentially a post-mitochondrial fraction, contains about 412.86 units of the enzyme per gfw of 18 h imbibed pea cotyledons, one unit being the amount of enzyme protein in mgs that liberates 1 u mole of paranitrophenol (pNP) or orthophosphate from paranitrophenyl phosphate (pNPP) per min under enzyme assay conditions. Ten percent of this activity was associated with the microsomal fraction containing largely plasma membrane, Golgi apparatus and endoplasmic reticulum. The remainder was mostly localized in the cytosol. Whether the particulate enzyme and cytosolic enzyme represent the same or modified form of the enzyme or are entirely different enzymes, is not known at present, although the specific activity of both microsomal and cytosolic APases are comparable.

The results shown in Table VI indicate that the PM fraction accounts for more than one third of the total APase activity in the crude microsomes. Golgi apparatus and ER account for about 4.5 and 6.4 percent of microsomal APase activity respectively. Nearly half the activity of the microsomal APase was lost during the isolation of various endomembrane fractions.

TABLE V : Distribution of acid phosphatase in various centrifugal fractions of pea cotyledons

Imbibed (18 h) pea cotyledons (100 g fw) were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 3 mM EDTA, 0.1 percent 2-mercaptoethanol, and subfractionated by differential centrifugation as described in the text. APase, acid phosphatase, pNP, paranitrophenol.

Fraction	Total protein (mg), ±SD	APase activity		Relative abundance[a] (percentage of total)
		Total (µMpNP/min), ±SD	Specific µMpNP/min/mg protein, ±SD	
12,000 xg supernatant	3855±380[b]	41286±4800	10.70±2.0	100.0
12,000-105,000 xg pellet	328±41	4222±192	12.87±2.3	10.2
105,000 xg supernatant	3090±410	34280±11200	11.09±2.0	83.0

a Total APase activity in 12,000 xg supernatant fraction was used as 100 percent for the purpose of calculating the percent relative abundance.

b ± Standard deviation of the average values of three or more experiments.

TABLE VI : Distribution of acid phosphatase in various membrane fractions

The crude microsomal fraction (12,000 - 105,000 xg pellet) was subfractionated by discontinuous sucrose density gradient centrifugation as described in the text. APase, acid phosphatase, PM, plasma membrane, GA, Golgi apparatus, SER, smooth endoplasmic reticulum.

Fraction	Total Protein (mg), ±SD	APase activity		Relative abundance (percentage of particulate)
		Total activity (μM pNP/min)	Specific activity (μM pNP/min/mg protein)	
12,000-105,000 xg pellet (crude microsomes)	328.0±12[a]	4221	12.87	100.0
PM	36.0±5	1504	41.77	35.6
GA	7.5±2	190	25.28	4.5
SER	9.5±3	271	28.50	6.4

a ± Standard deviation of the average values of three or more experiments.

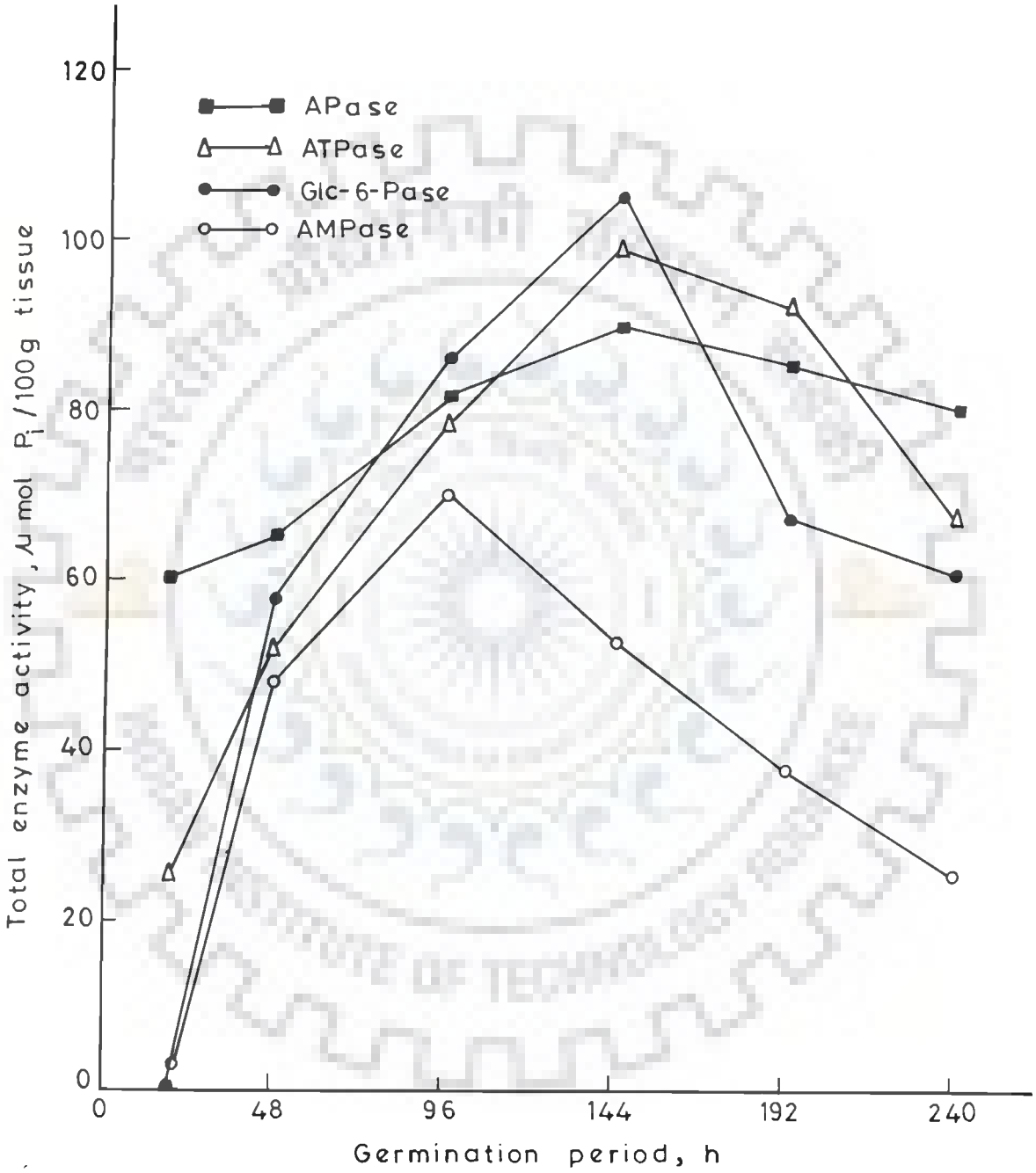
Nevertheless 46% of recoverey is considered quite good since the PM, GA and ER fractions are apparently free from the cross contamination. For the sake of purity some membranes had to be sacrificed. In addition some activity was associated with the fluffy material which was discarded. The PM-APase shows the highest specific activity, which is about 3.2 times of the crude microsomal APase, followed by GA and ER.

4.3 Activity levels of some pea cotyledons microsomal ortho-phosphoric monoester phosphohydrolases as a function of germination period

The variation in the levels of activity of some orthophosphoric monoester phosphohydrolases associated with the microsomal membrane fraction (12,000 - 105,000 x g pellet) of pea cotyledons during 240 h germination is shown in Fig 4.3. The monoester phosphohydrolase activity measured with P-nitrophenyl phosphate (pNPP) as substrate was referred to as acid phosphatase (E.C. 3.1.3.2). While 5´-adenosine monophosphatase (AMPase) exhibited maximal activity after 96 h, all other phosphatases, namely glucose-6-phosphatase, ATPase and acid phosphatase (APase) showed peak activity on the sixth day of germination. At this physiological stage, the activity of pea cotyledon microsomal glucose-6-phosphatase, ATPase and APase were nearly comparable. From these results it was apparent that 48 h or longer period, germinating pea cotyledons would, in addition to APase, contain almost equal amounts of glucose-6-phosphatase, AMPase and ATPase which are likely to interfere in the purification and actual

FIG 4.3 : **Activity of some microsomal acid phosphatases of pea cotyledons as a function of germination**

The microsomal (12,000 - 105,000 x g) fraction (100 - 200 ug protein) was incubated with 1 mM substrate in 50 mM sodium acetate buffer, pH 5.0, in a final volume of 1.5 ml for 30 min at 30°C. The reaction was terminated by the addition of 0.5 ml of 20% cold trichloroacetic acid. The denatured proteins were removed by centrifugation for 10 min at 5,000 xg and the Pi in the supernatant was determined by the method of Fiske-SubbaRow (82). Results were expressed as uM Pi/100 g tissue.


APase
ATPase
Glc-6-Pase
AMPase
Total enzyme activity, µ mol P_i /100g tissue
Germination period, h
0
20
40
60
80
100
120
0
48
96
144
192
240

measurement of APase activity. However, at 18 h after imbibition, while the APase activity was substantial, the AMPase and glucose-6-phosphatase activities were virtually absent. Further, the ATPase activity was also markedly lower, less than half the APase. In other words, by using pea cotyledons after 12 - 18 h imbibition, it was possible to prepare a microsomal fraction of sufficiently high APase activity but at the same time low in ATPase activity and nearly completely devoid of AMPase and glucose-6-phosphatase activities .

4.4 Relative abundance of some common phosphatases in pea cotyledon membranes

Table VII shows the relative levels of activities of some common phosphatases in PM, GA and ER fractions isolated from pea cotyledons after 18 h imbibition in water at room temperature ($25^{o}C$). Of the various phosphatases tested, the enzyme assayed using pNPP as substrate (referred to as acid phosphatase, APase) was most active in all three membrane fractions. This was followed by ATPase, glyceraldehyde-3-phosphatase, glucose-6-phsophatase and 5´-mononucleotidase (AMPase). Among the various membranes, the APase and ATPase showed the highest abundance in PM, followed by ER and GA. The level of glucose-6-phosphatase, glyceraldehyde-3-phosphatase and AMPase was extremely low in PM. Hence, except ATPase, inteference from other acid phosphatases will be relatively very low and PM may be suitable for isolation of APase.

TABLE VII: Relative activities of some common acid phosphatases in the membrane fractions of pea cotyledons

PM, GA and ER membrane fractions were prepared from the pea cotyledons as described under `Methods´. Membrane fraction (0.1 mg protein) was incubated with 1 mM substrate in 50 mM sodium acetate buffer,pH 5.0, in a final volume of 1.5 ml for 30 min at 30°C. After incubation, the reaction was terminated by the addition of 0.5 ml of 20% cold trichloroacetic acid. The denatured proteins were removed by centrifugation and the Pi in the supernatant was determined by the method of Fiske-SubbaRow (82). The enzyme activity measured with p-nitrophenyl phosphate as substrate, referred to as APase, was used as 100 percent.

Substrate used[a]	Phosphatase activity in membrane fraction					
	µmol Pi/min/mg protein			% relative activity		
	PM	GA	ER	PM	GA	ER
pNPP	8.60	3.30	3.50	100.0	100.0	100.0
Glc-6-p	0.20	0.35	0.44	2.3	10.6	12.6
Gly-3-p	0.86	0.40	0.49	10.0	12.1	14.0
AMP	0.30	0.00	0.00	3.5	0.0	0.0
ATP	4.60	2.00	2.60	53.5	60.6	74.3

a pNPP, p-nitrophenyl phosphate, Glc-6-p, glucose-6-phosphate, Gly-3-p, glyceraldehyde-3-phosphate; AMP, 5´-adenosine monophosphate; ATP, 5´-adenosine triphosphate

4.5 Some properties of membrane-associated acid phosphatase

4.5.1 Kinetic properties and optimum pH

In order to purify the membrane-bound acid phosphatase, some properties of PM-,GA- and ER-associated APase were investigated. The activity of PM-, GA- and ER-associated APase was linear with respect to time (Fig 4.4) and protein concentration (Fig 4.5) for at least 30 min and 300 µg, respectively. In fact in case of GA-APase and ER-APase the reaction was linear upto 60 min.

The PM-, GA- and ER-APase exhibited identical optimum pH range from 5.25 to 5.75 (Fig 4.6). Below and above this pH range, the activity of membrane associated APases declined sharply.

The apparent Km values of the PM-,GA- and ER-APases determined according to Lineweaver-Burk plots (Fig 4.7) using P-nitrophenyl phosphate as substrate, were approximately 500 µM, 500 µM and 310 µM, respectively. The V_{max} values for these enzymes were 66.7 uM, 28.6 uM and 33.3 µM per min per mg protein. These data show that whereas the Km values of PM-,GA-, and ER-associated enzymes are comparable, the V_{max} of PM - APase is about 2 times compared to that of GA and ER enzymes.

4.5.2 Stability at 0 - 4^{o}C

The stability of the membrane-bound enzymes was tested with a view to purify the enzyme. The membrane fractions were

FIG 4.4 : Time course of the membrane-associated APase

The enzyme activity was measured by the standard assay system described under `Methods´, using a constant amount of protein (80 µg) and incubated for indicated periods.

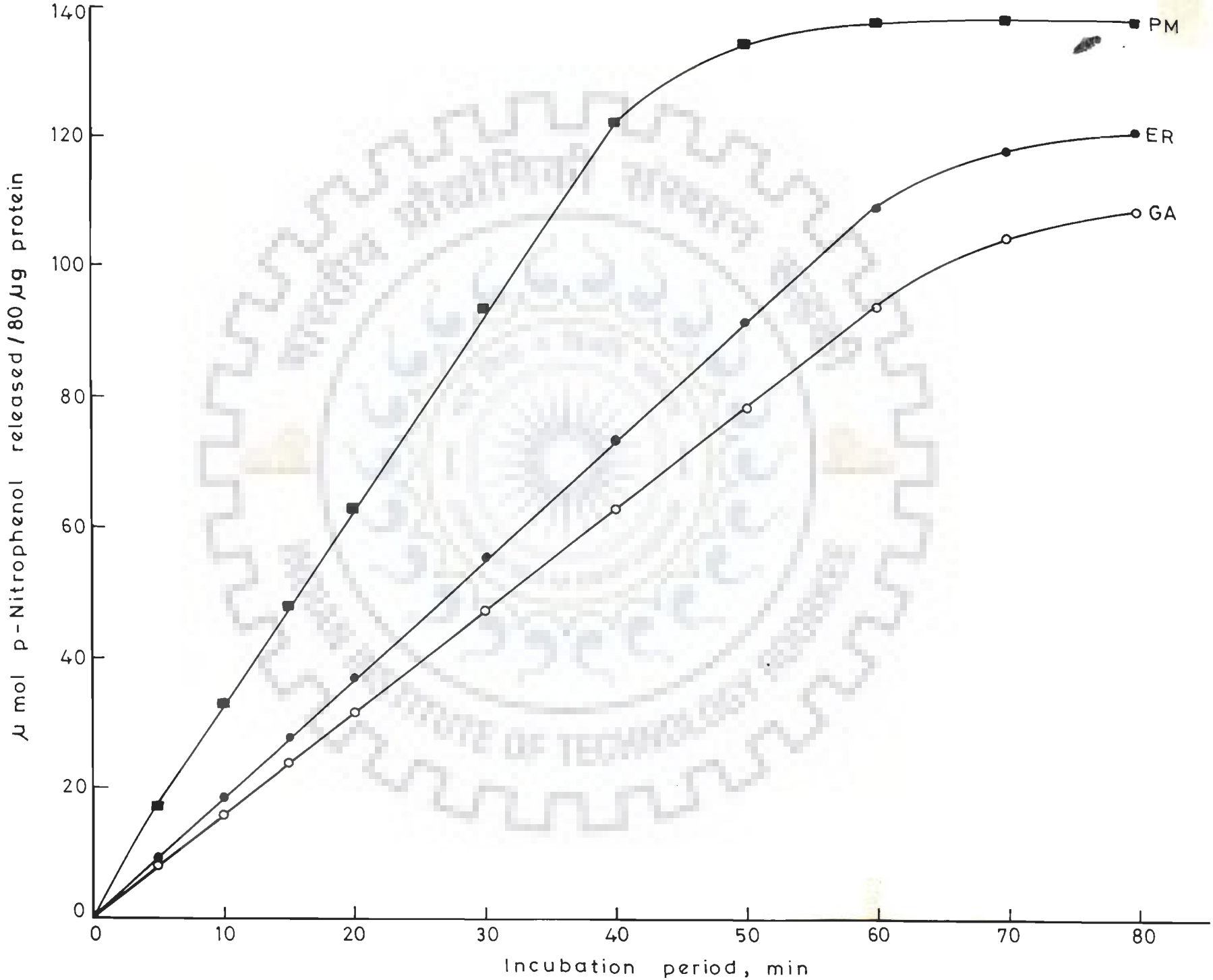

140
120
100
80
60
40
20
0
μ mol p-Nitrophenol released / 80 μg protein
0
10
20
30
40
50
60
70
80
Incubation period, min
PM
ER
GA

FIG 4.5 : **Protein concentration dependence of membrane-bound APase at pH 5.0, from various endomembrane fractions.**

The enzyme activity was determined by the standard assay system described in `Methods´. The enzyme was incubated for 15 min (PM) and 30 min (GA and ER) with indicated protein concentrations.

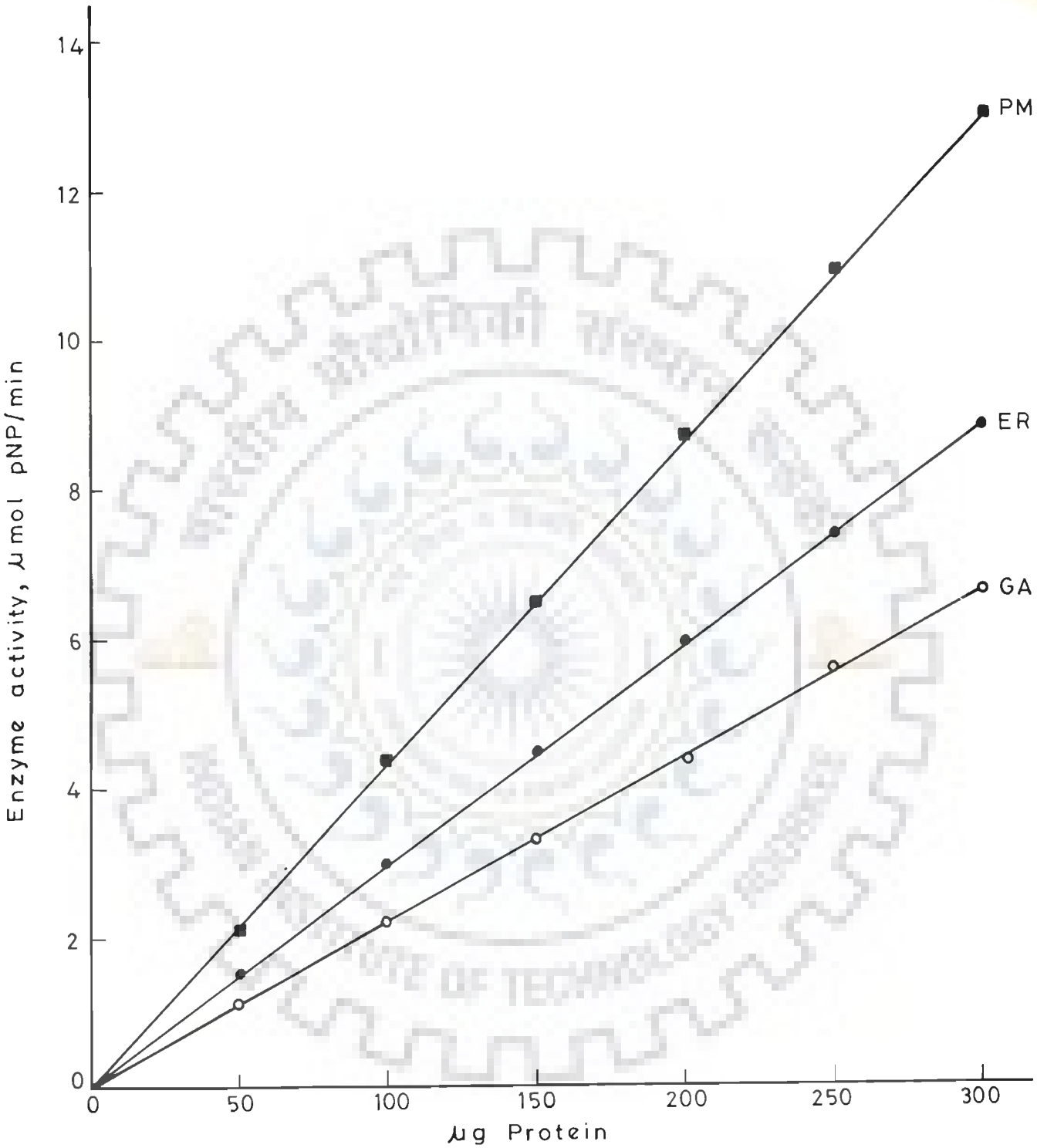

Enzyme activity, µmol pNP/min
µg Protein
PM
ER
GA
0
2
4
6
8
10
12
14
0
50
100
150
200
250
300

FIG 4.6 : Optimum pH profiles of pea cotyledons PM-, GA- and ER-associated APases

The PM, GA and ER were prepared as described in text. The enzyme activity was determined by the standard assay system except that the buffer and pH varied. The buffers used were 50 mM acetate buffer, pH 3.6 - 6.4 and 50 mM Tris-HCl, pH 6.8 - 8.

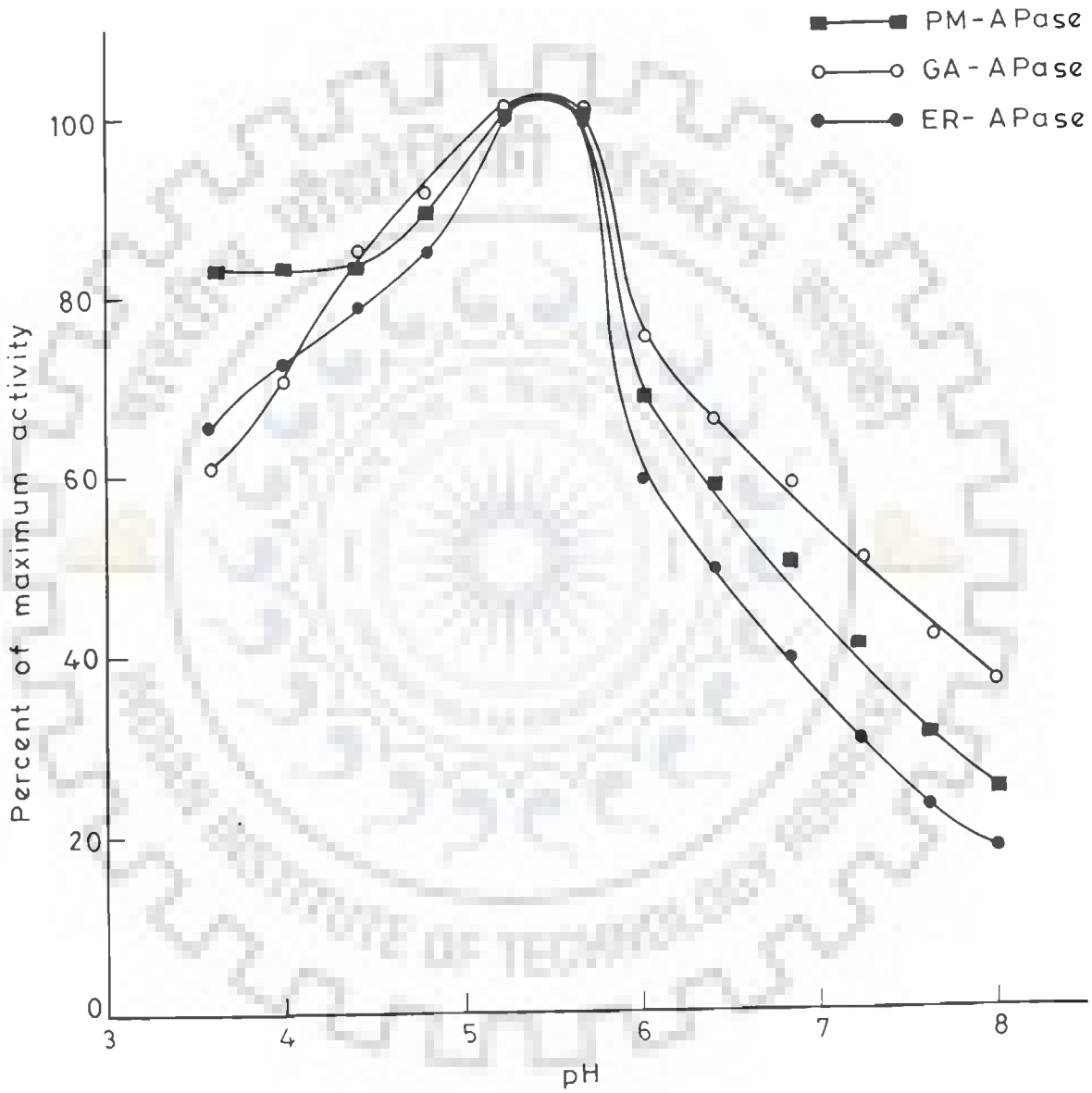

PM-APase
GA-APase
ER-APase
Percent of maximum activity
100
80
60
40
20
0
3
4
5
6
7
8
pH

FIG 4.7 : **Lineweaver-Burk plots of membrane-bound APase of pea cotyledons**

The PM, GA and ER membranes were prepared as described in text. Standard assays with varying amounts of substrate were carried out for 15 min for PM and 30 min for GA and ER with 0.1 mg protein. Km and V_{max} values were calculated from the intercepts on X-axis and Y-axis respectively.

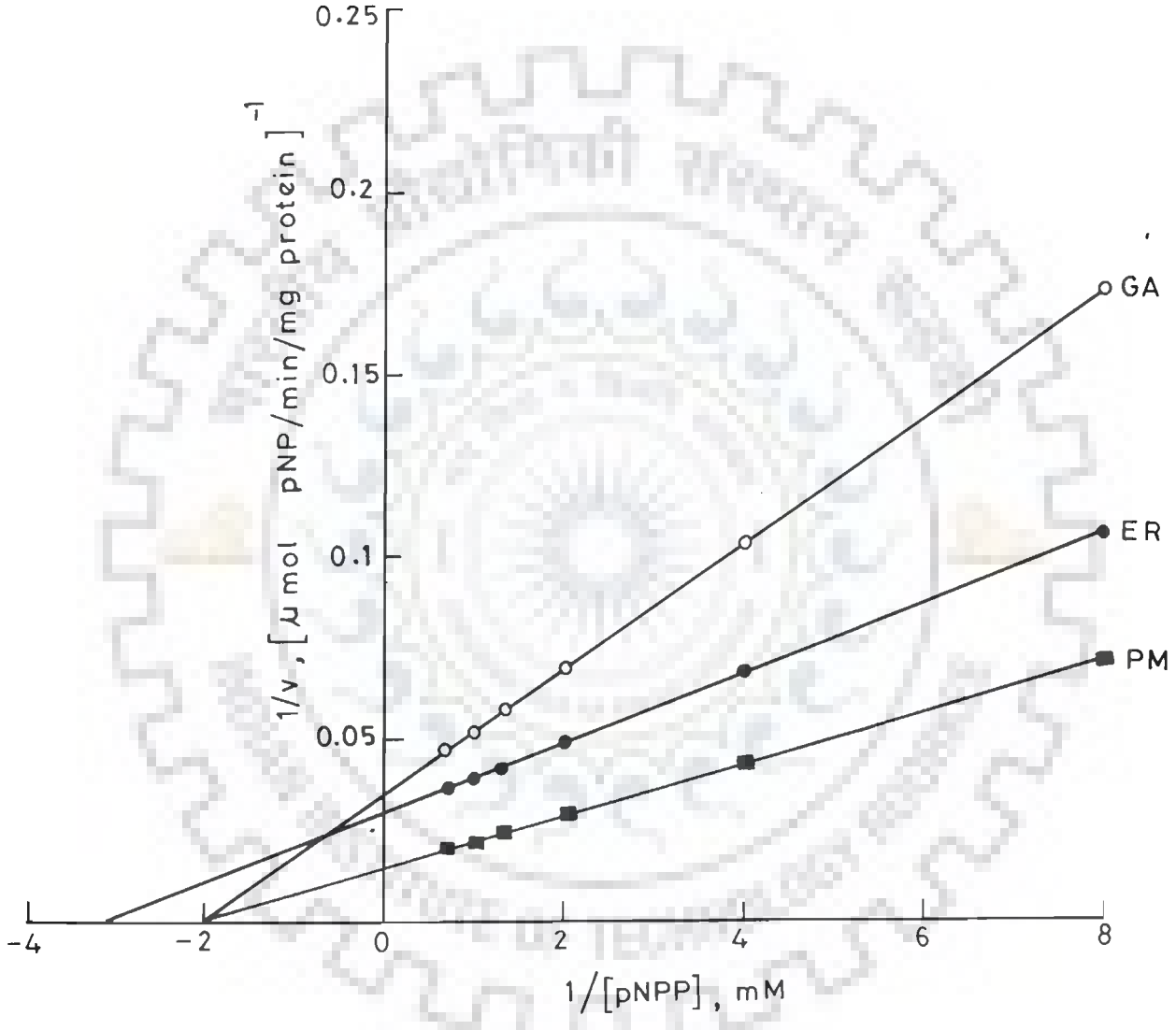

0.25
0.2
0.15
0.1
0.05
1/v, [μ mol pNP/min/mg protein]⁻¹
GA
ER
PM
-4
-2
0
2
4
6
8
1/[pNPP], mM

TABLE VIII : Satbility of membrane-associated acid phosphatase

The membrane fractions were stored in 50 mM Tris-HCl buffer, pH 7.4, containing 0.10 percent 2-mercaptoethanol and 250 mM sucrose at 0 - 4°C for indicated period of storage. The enzyme activity was assayed as described in `Methods´.

Period of storage (days)	Residual Enzyme Activity					
	PM-APase		GA-APase		ER-APase	
	µMpNP/min/ mg protein	%	µMpNP/min/ mg protein	%	µMpNP/min/ mg protein	%
0	38.6	100.0	22.5	100.0	25.2	100.0
1	38.2	99.0	23.1	102.7	25.2	100.2
2	39.0	102.6	22.2	98.7	24.6	97.6
3	38.2	99.6	18.9	84.0	20.1	79.8
4	38.6	100.0	12.6	56.0	9.3	36.9
5	40.0	103.6	6.3	28.0	4.8	19.6
6	38.4	99.5	2.4	10.7	1.8	7.1
7	38.6	100.0	0.9	4.0	0.6	2.4
15	38.0	98.4	0.0	0.0	0.0	0.0
30	37.1	96.1				

stored at 0-4°C in the suspension buffer and the APase activity was assayed by the standard procedure. Results are shown in Table VIII. Surprisingly, the PM-APase was fully stable under the storage conditions indicated, for 1 month without any apparent loss of activity. The GA- and ER-APases, however, were rendered totally inactive in one week, but about 80% activity was retained upto 72 h storage at 0 - 4°C. Thus, PM-,GA- and ER-APases show differential low temperature stabilities and suggest some kind of post-translational modification which might be responsible for greater stability of the PM-APase than that of GA- and ER-APases. However, if the relatively greater stability is a characteristic property of the PM enzyme, it is uncertain at the moment. Nevertheless, the PM-APase appears to be a suitable candidate for the purification work.

4.5.3 Effect of some biologically active cations and anions on the membrane-associated APase.

Table IX summarizes the effect of some monovalent and divalent metal ions, F^-, PO_4^{3-}, $Mo_7O_{24}^{6-}$, citrate, tartarate and EDTA on the PM-APase, GA-APase and ER-APase of pea cotyledons, at a final concentration of 10 mM. Addition of EDTA to the enzyme assay mixture did not result in loss of activity. In fact about 25% activation of PM-APase activity was observed. For GA-APase and ER-APase, the activation was about 15 to 18% respectively. These results indicate that there is no metal ion requirement for APase activity. Na^+ and K^+ do not have any effect either. These results together with EDTA results rule out the presence of

TABLE IX : The effect of some common biologically active cations and anions on membrane-associated APase

The APase activity was measured using pNPP as substrate as described under `Methods´ without and with additions of ions. Enzyme activity without additions was used as 100 percent.

Additions	APase activity (µM pNP/min/mg protein)			% Relative activity		
	PM	GA	ER	PM	GA	ER
None	43.2	26.1	28.5	100.0	100.0	100.0
EDTA	55.3	30.3	33.7	128.0	116.0	118.0
Na^{+}	42.5	26.1	28.0	98.4	100.0	98.0
K^{+}	43.2	26.1	28.0	100.0	100.0	100.0
Mg^{2+}	27.7	11.7	12.0	64.0	45.0	42.0
Ca^{2+}	39.5	14.9	9.4	91.0	57.0	33.0
Mn^{2+}	16.7	13.9	10.3	39.0	53.0	36.0
Zn^{2+}	3.5	2.6	2.9	8.0	10.0	10.0
Hg^{2+}						
Cu^{2+}	4.3	5.5	6.7	10.0	21.0	23.5
Ni^{2+}	24.7	4.4	8.7	57.0	17.0	30.5
F^{-}	1.5	1.3	1.0	3.0	5.0	3.5
Citrate	184.0	36.6	63.7	426.0	140.0	223.5
Tartarate	162.0	32.9	62.7	375.0	126.0	220.0
PO_4^{3-}						
$Mo_7O_{24}^{6-}$						

Na^+/K^+ activated ATPase also. Most of the divalent metal ions tested (Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}) have inhibitory effect on the PM-, GA- and ER-APases, although the degree of inhibition differ markedly. Zn^{2+}, Mn^{2+}, Hg^{2+} and Cu^{2+} are highly potent inhibitors of membrane-associated APases. There is also differential inhibitory effect of metal ions on PM-, GA- and ER-APases. For example, there is little inhibition of PM-APase by Ca^{2+}, but the GA-APase and ER-APase are inhibited by 43 and 67% respectively. Ni^{2+} inhibits GA-APase to 83%, while under identical conditions, PM-APase and ER-APase are inhibited upto 43 and 69.5%. Thus besides resemblance, the PM-, GA- and ER-associated enzymes also show distinct differences. In order to avoid interference of EDTA in the enzyme activity, assays are carried out in the absence of EDTA. Flouride ions are also strong inhibitors of the membrane-bound enzyme and the potency of inhibition among the various membrane-bound enzymes is comparable. Complete inhibition of the PM-, GA- and ER-associated enzymes by phosphate ions, a product of the enzyme reaction, and molybdate at this concentration is observed. Varying degrees of activation by citrate and tartarate ions are observed for the PM-, GA- and ER-bound APases. Whether the activation by citrate and tartrate is a characteristic property of the membrane-bound APase or due to some other protein is not known at the moment.

4.6 Pruification of the plasma membrane acid phosphatase

Fig 4.8 shows the flow chart of the purification and separation protocols of APase isoenzymes from the plasma

FIG 4.8 : Flow chart of the purification and separation protocols of APase isoenzymes (APase I and APase II) from plasma membranes of pea cotyledons

Pea cotyledons (18 h imbibed, 200 g fresh weight)

1. Homogenization in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 3 mM EDTA and 0.1% 2- mercaptoethanol.

2. Filtration through 4 layers of cheese cloth and centrifugation sequentially at 12,000 xg for 20 min and 105,000 xg for 60 min.

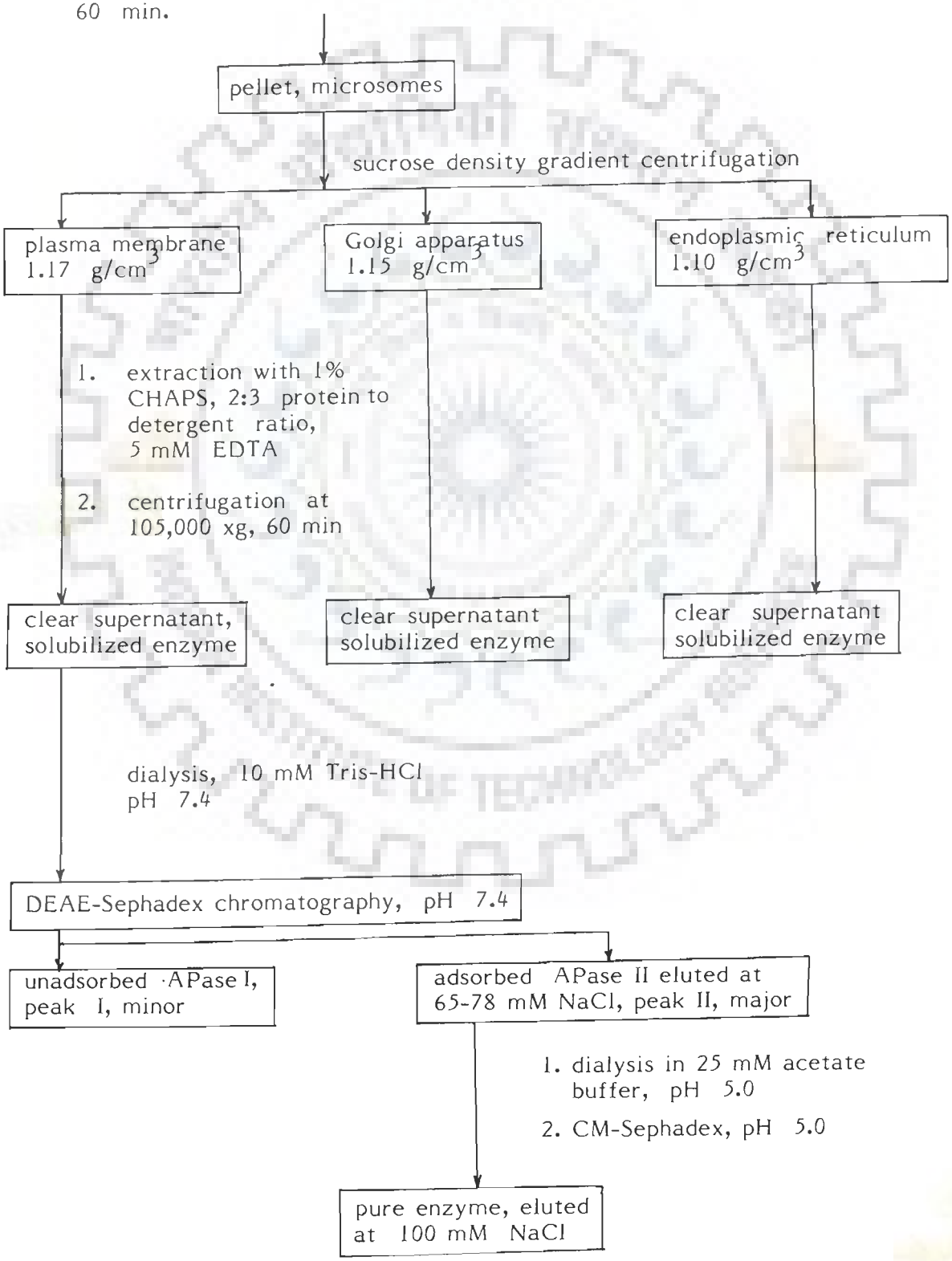


TABLE X : Purification of pea cotyledon plasma membrane-associated acid phosphatase isoenzymes

The plasma membrane fraction, apparently free from GA and ER membrane, was obtained from the crude particulate fraction (12,000-105,000 xg) as described under `Methods´. The purified PM was extracted with 1% CHAPS in the presence of 5 mM EDTA, followed by 105,000 xg centrifugation as described in text. The supernatant fraction representing the solubilized enzyme was dialyzed and chromatographed as described under `Methods´. The purification data were computed relative to the crude microsomal fraction.

Sl. No.	Step	Total protein (mg)	Total APase activity units x 10^3	Specific activity of APase units/mg	Purification fold	Yield %
1.	Crude membrane fraction 12,000-105,000xg pellet)	750.000	10.99	14.65	1.0	100.0
2.	Plasma membrane	80.000	3.53	44.10	3.0	32.1
3.	CHAPS extract (105,000 xg supernatant)	26.000	3.02	116.15	7.9	27.5
4.	DEAE-Sephadex peak I (unadsorbed protein, APase I)	2.400	0.21	87.50	5.9	1.9
	Peak II (adsorbed protein, APase II)	3.200	1.84	575.00	39.2	16.7
5.	Dialysis, pH 5.0 and centrifugation,supernatant	2.100	1.68	800.00	54.6	15.3
6.	CM-Sephadex APase II	0.085	0.12	1403.20	95.8	1.1

membranes of pea cotyledons and Table X summarizes the purification data. The use of 18 h imbibed pea cotyledons for the preparation of total microsomal membrane fraction that was almost devoid of 5′-nucleotidase activity and relatively low in ATPase activity (Fig 4.3) was a crucial step in the purification of the APase, as it was difficult to remove the contamination of 5′-nuceotidases, especially when they also exhibit the properties of APase (46) including the solubilization characteristics (233).

Fractionation of the PM from the GA and ER by sucrose density gradient resulted in three fold purification of acid phosphatase. The next step involved the use of a suitable detergent that would solubilize the membrane-bound enzyme in both active and stable form. The effect of six different detergents on the activity of the plasma membrane-associated APase is shown in Table XI. Brij-35, deoxycholate and taurodeoxycholate were inhibitory particularly at high concentrations, taurodeoxycholate being the strongest. Triton X-100, Nonidet NP-40 and CHAPS appeared to be good candidates for the solubilization of PM-APase, since they did not have any adverse effect on the APase activity. In fact, at all concentrations (0.1% to 1.0%), a significant enhancement of the enzyme activity was observed. Of these detergents, however, CHAPS was preferred because of its much higher critical micelle concentration (CMC, 0.49 mM) than Triton X-100 (CMC 0.02 mM) or Nonidet NP-40 (108). Since detergent concentration and protein-to-detergent ratio are often critical in solubilizing the membrane-bound enzyme in active and

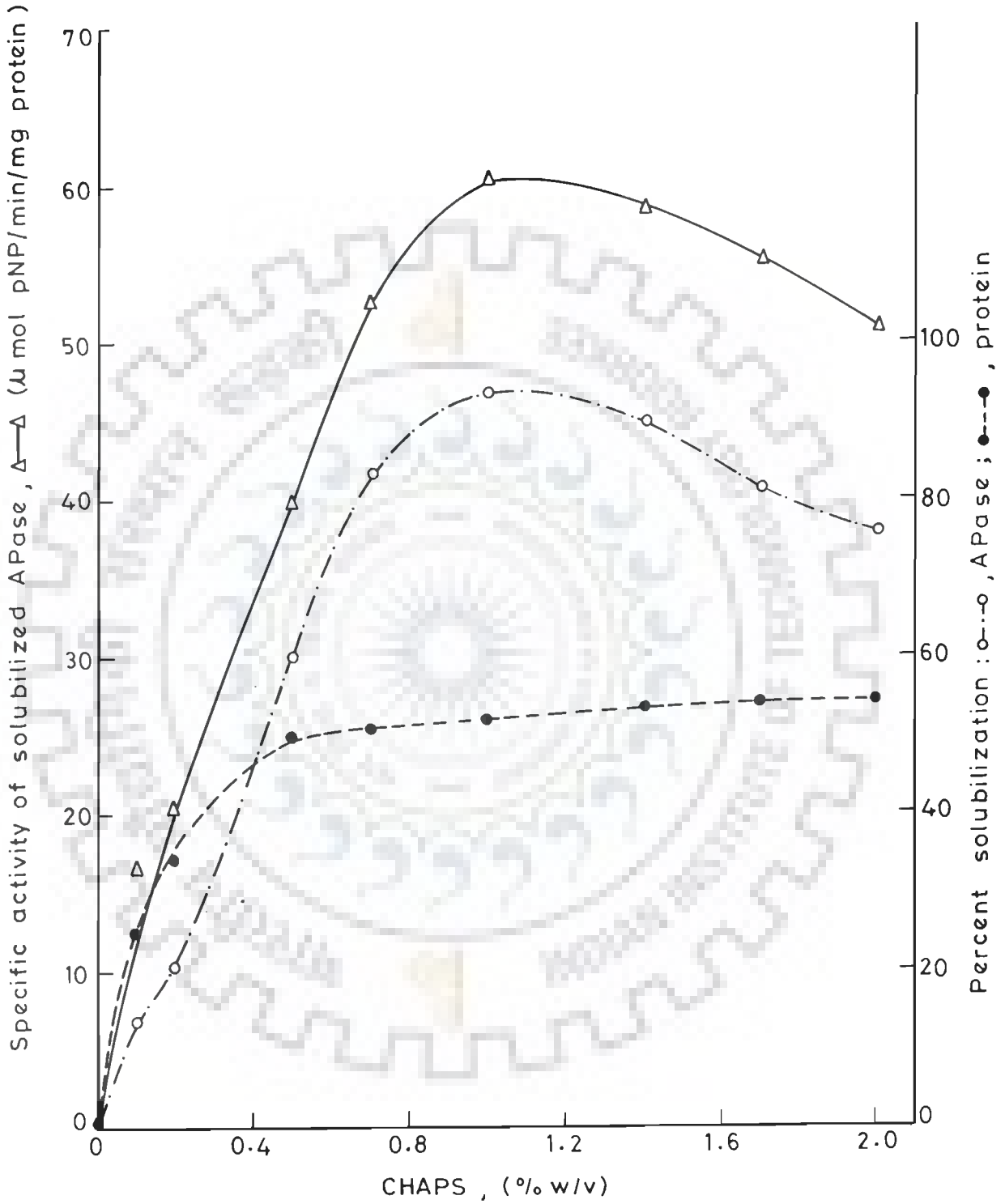
TABLE XI : Effect of various detergents on the activity of plasma membrane-associated APase from pea cotyledons

The enzyme assays were carried out without and with indicated amounts of detergent as described in `Methods´.

Detergent concentration	Detergent and APase activity, µmol pNP/min/mg protein					
	Triton X-100	CHAPS	Nonidet PN-40	Brij-35	deoxy-cholate	Tauro-cholate
0.0	33.3	33.3	33.3	33.3	33.3	33.3
0.1	35.6	47.2	32.2	31.0	33.3	32.0
0.3	40.0	47.3	40.2	29.2	31.3	21.2
0.5	45.2	47.1	43.4	28.5	29.2	16.7
0.8	46.7	47.5	45.6	26.0	26.6	10.2
1.0	46.0	47.0	46.2	21.5	20.0	6.4

FIG 4.9 : **Solubilization of APase activity as a function of detergent concentration at a fixed protein concentration (2.7 mg/ml)**

The plasma membrane, in 50 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 0.1% 2-mercaptoethanol and varying concentrations of CHAPS in a final volume of 6 ml, was homogenized on a Vortex for 1 x 4 min followed by 30 min incubation in ice. After centrifugation of the homogenate at 105,000 xg for 60 min, the supernatant was carefully removed with a Pasteur pipette and referred to as solubilized enzyme. The protein content and APase activity were determined by the standard assay methods described under `Methods´. Results were expressed as specific activity of solubilized and percent of activity and protein solubilized.


Specific activity of solubilized APase, Δ—Δ (μ mol pNP/min/mg protein)
Percent solubilization : o---o, APase ; ●---●, protein
CHAPS, (% w/v)
0
10
20
30
40
50
60
70
0
20
40
60
80
100
0
0.4
0.8
1.2
1.6
2.0

stable form (108), the solubilization of APase activity from the PM as a function of the detergent concentration as well as protein-to-detergent ratio, was investigated. It was found that (Fig 4.9) at a fixed protein concentration (2.7 mg/ml), in the present case, the solubilization of APase activity from the plasma membranes increased rapidly with increasing concentration of CHAPS from 0 to 1%; thereafter the percent solubilization started declining indicating the inhibition of the APase activity by CHAPS at a final concentration greater than 1 percent. In fact, at this concentration of CHAPS, both total and specific activities of the solubilized enzyme were maximum with about 95% of the PM-APase liberated from the membranes. Interestingly, the percent solubilization of protein remained almost constant, varying from 50 to 55% only. Thus, it seems that CHAPS affects the selective solubilization of APase from the PM.

The effect of detergent concentration in terms of protein-to-detergent ratio on the activity and solubilization of the enzyme is shown in Fig 4.10. In this experiment, the final detergent concentration was maintained at 1% and the protein concentration was varied as to obtain 1:1, 1:1.5, 1:2 and 1:3 protein-to-detergent ratios. The results (Fig 4.10) indicated that a protein-to-detergent ratio of 2:3 is optimum for the solubilization of APase from the pea cotyledon PM. At this detergent concentration and protein-to-detergent ratio, nearly 85% enzyme activity and about 50% protein of PM were solubilized.

The effect of different concentrations of EDTA on the

FIG 4.10 : **Solubilization of PM-APase activity as a function of protein-to-detergent ratio at a fixed detergent concentration of 1%.**

The protein-to-detergent ratio was adjusted to 1:1, 1:1.5, 1:2 and 1:3 maintaining the final concentration of detergent at 1%. Solubilization was carried out as described under `Methods´. The results were expressed as specific activity of soluble APase, percent of total activity and protein solubilized.

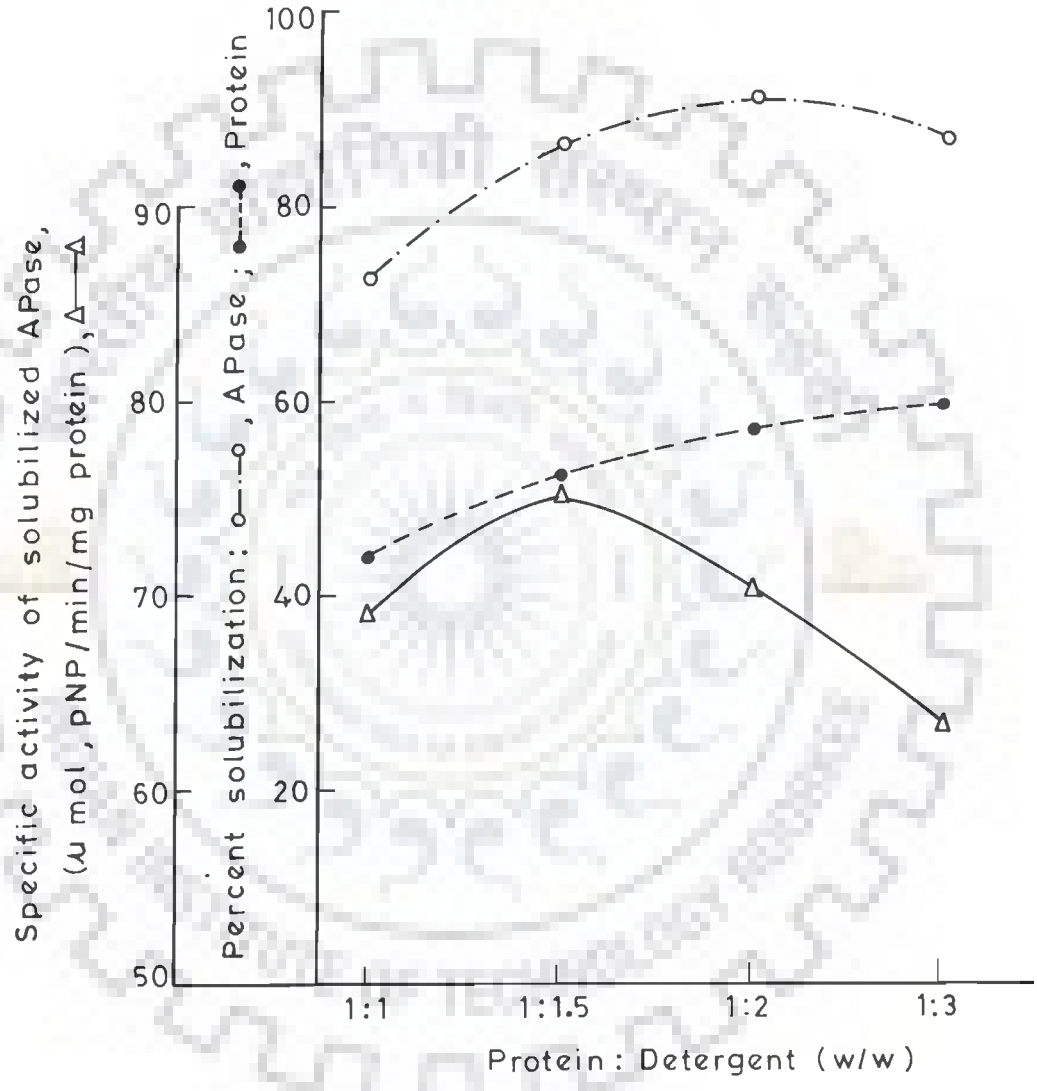
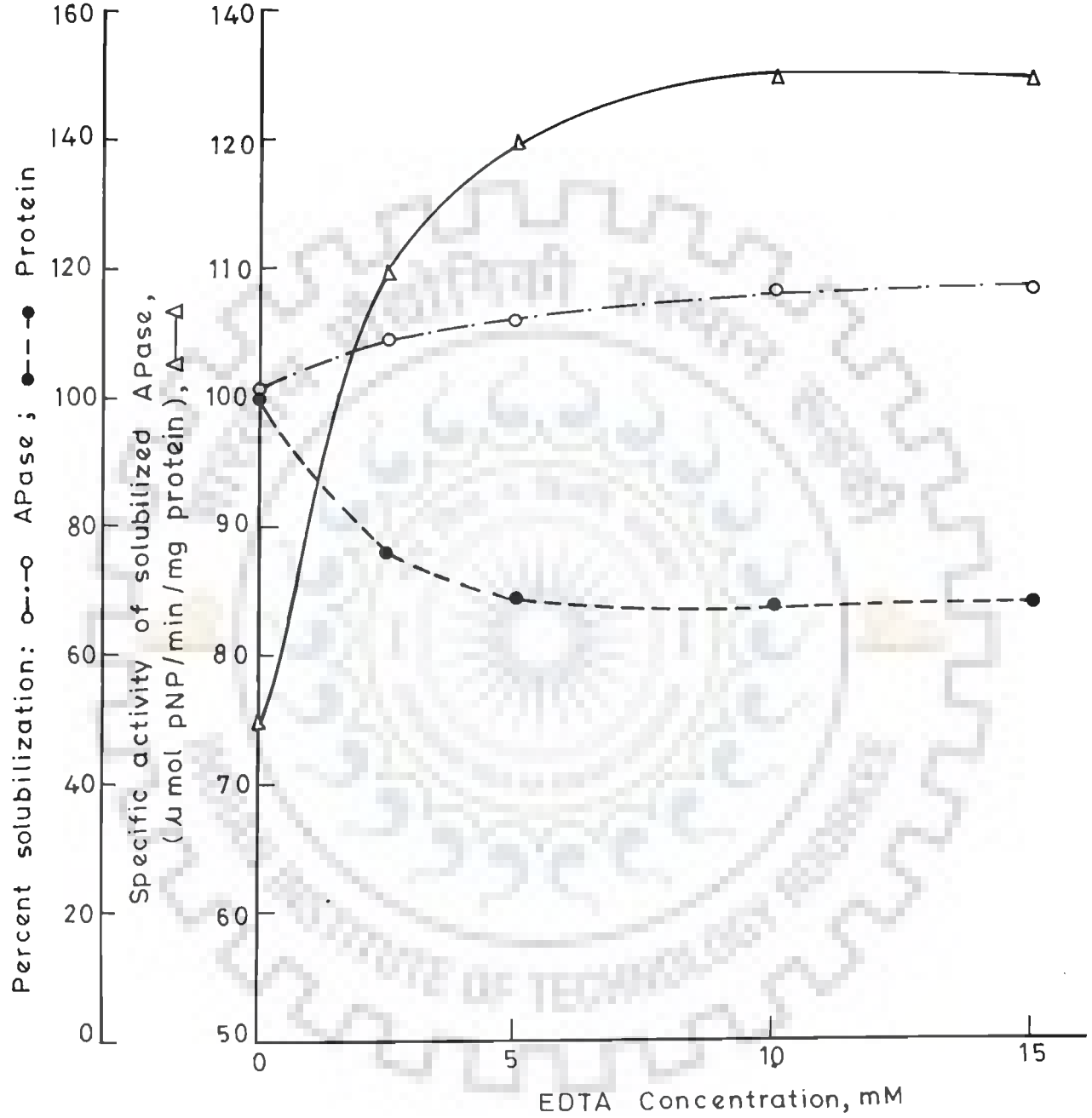

Specific activity of solubilized ATPase,
(μ mol, pNP/min/mg protein), Δ—Δ
Percent solubilization: o—o, ATPase; •---•, Protein
100
90
80
70
60
50
80
60
40
20
1:1
1:1.5
1:2
1:3
Protein : Detergent (w/w)

FIG 4.11 : Solubilization of PM-APase activity as a function of EDTA concentration at a fixed protein-to-detergent ratio (2 : 3) and constant CHAPS concentration (1%)

The plasma membrane fraction (20 mg protein/ml) was extracted with 1% CHAPS and protein-to-detergent ratio of 2:3 in the absence and presence of different concentrations of EDTA as described in `Methods´. The homogenate was centrifuged at 105,000 xg for 60 min. The supernatant was used as solubilized enzyme. Enzyme activity and protein were assayed as in text. The results were expressed as specific activity of soluble APase and percent of total activity and protein solubilized.


Percent solubilization: o---o APase ; •---• Protein
Specific activity of solubilized APase, (μ mol pNP/min/mg protein), Δ—Δ
160
140
120
100
80
60
40
20
0
140
120
110
100
90
80
70
60
50
0
5
10
15
EDTA Concentration, mM

activity and solubilization of the enzyme is shown in Fig 4.11. In this experiment, the final concentration of the detergent (1%) and the protein-to-detergent ratio (2:3) were maintained constant whereas the final concentration of EDTA varied from 2.5 to 15 mM. The results indicated that EDTA had a profound effect on the solubilization of the enzyme by selectively suppressing the solubilization of some proteins other than APase although the variations were relatively very small. From 5 mM EDTA upwards the solubilized proteins were suppressed from 55% to about 35% whereas the specific activity increased to about 60% over that in its absence. In terms of total activity solubilized, however, the increase was only 10%. Hence 5 mM EDTA was included in the solubilization system. The specific activity of the solubilized enzyme was increased to 1.6 fold over control and about 8 fold over the crude microsomal APase. The solubilized enzyme was fairly stable and could be stored for about 30 days at - 20^{o}C and for about a week at 0 - 4^{o}C without any significant loss of activity.

The next step of purification of PM-APase involved DEAE-Sephadex column chromatography. Fig 4.12 illustrates the elution profile of dialyzed CHAPS-solubilized enzyme on a DEAE-Sephadex column. Nearly 90% of the APase activity applied on the column was absorbed on DEAE-Sephadex and only a small amount (less than 10%) was not retained on the column. This 10% enzyme activity which eluted in the void volume was not due to over-loading of the column, but appeared to be an isoenzyme of APase as it

FIG 4.12 : **DEAE-Sephadex column chromatography of CHAPS-solubilized acid phosphatase from plasma membranes of pea cotyledons**

The solubilized enzyme was dialyzed in 10 mM Tris-HCl, pH 7.4, and loaded (12 ml) on the pre-chilled, pre-equilibrated column (1 x 12 cm). The column was washed with the same buffer (25 ml) and the adsorbed proteins were eluted by a linear gradient of sodium chloride (0 - 250 mM) in the same buffer. Fractions (1.5 ml) were collected at a flow rate of 15 ml/h. Aliquots (0.1 ml) from every other fraction were assayed for APase activity and protein by the standard procedures. Fractions indicated by bar (46 - 51) were pooled and used for further studies.

o—o, ATPase activity, μ mol pNP/0.1 ml/15 min x 10
●—●, Protein, μg/0.1 ml
NaCl gradient 0–250 mM
NaCl mM
Fraction numbers (1.5 ml/frac.)
I
II
III
9–13
46–51
0
4
8
12
16
20
24
28
10
20
30
40
50
60
70
80
90
100
250
200
150
100
50

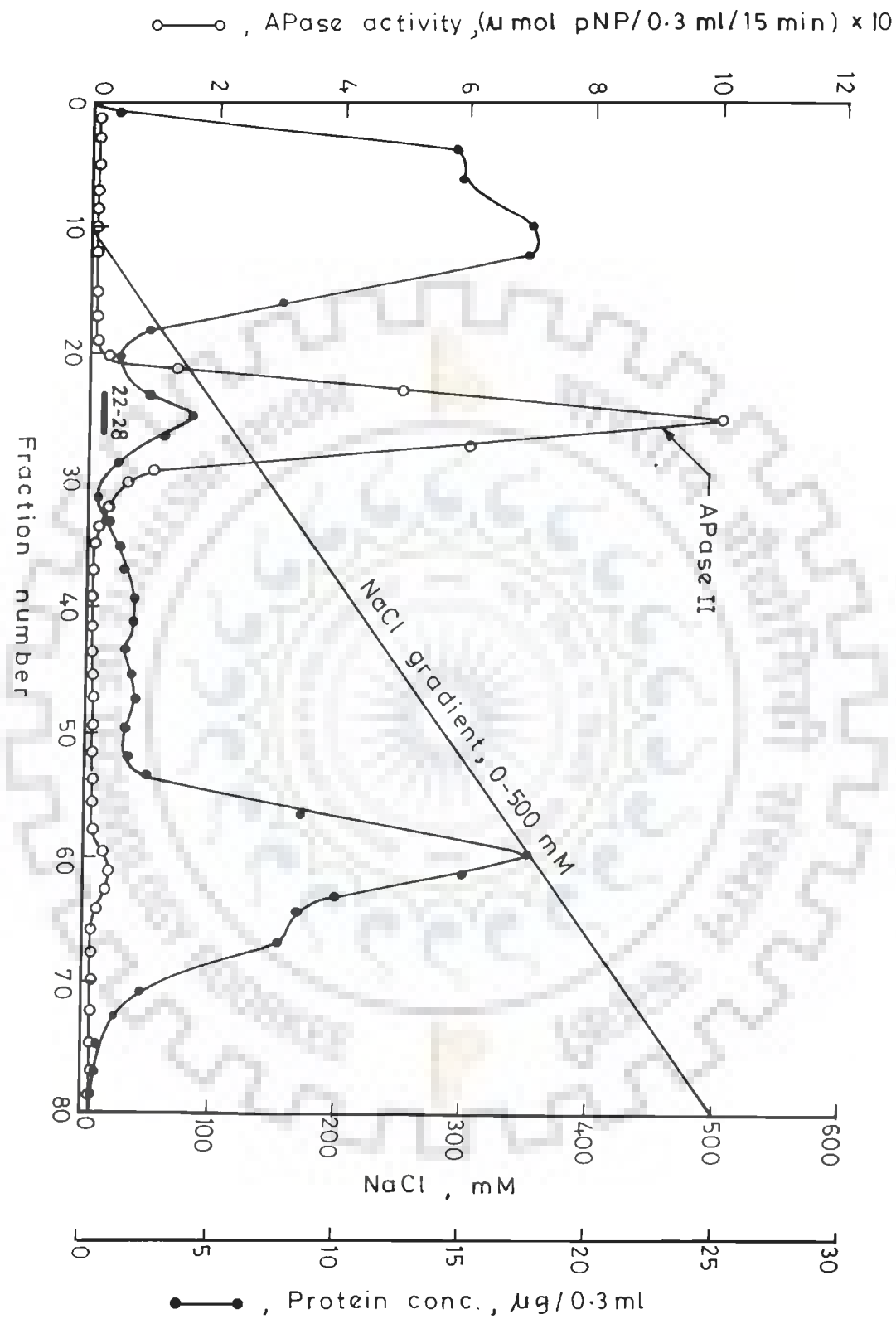
FIG 4.13 : Rechromatography of DEAE-Sephadex peak I (APase I) on DEAE-Sephadex

The active fractions of peak I (APase I) from the first column were pooled (8 ml) and applied to a second DEAE-Sephadex column (1 x 5 cm). Details of the run were exactly as described for the first column.

Washing with
equilibration buffer
I
APase I
II
14-17
NaCl gradient, 0-250 mM
o—o, Enzyme activity, μmol pNP/0·2 ml/15 min
•—•, Protein conc. μg/0·2 ml
NaCl, mM
Fraction number, 1·5 ml/frac
0
10
20
30
40
50
60
70
80
90
120
100
80
60
40
20
0
300
250
200
150
100
50

FIG 4.14 : **CM-Sephadex chromatography profile of DEAE-Sephadex peak II (APase II)**

The pooled fractions of peak II (APase II) from DEAE-Sephadex (10 ml) were dialyzed overnight against two changes of 1 litre 25 mM sodium acetate buffer, pH 5.0, containing 1 mM 2-mercaptoethanol, centrifuged for 5 min at 10,000xg to remove denatured protein and then loaded on pre-equilibrated CM-Sephadex column (1 x 5 cm). The column was washed with the same buffer (15 ml) and adsorbed proteins were eluted by a linear gradient of NaCl (0 - 500 mM). Fractions (1.5 ml) were collected at a flow rate of 15 ml/h. Aliquots (0.5 ml) from every other fraction were assayed for APase activity and protein by the standard methods. Fractions indicated by bar were pooled (22 - 28) and found to be pure by SDS - PAGE.


o——o , APase activity, (μ mol pNP/0·3 ml/15 min) × 10
0
2
4
6
8
10
12
Fraction number
0
10
20
30
40
50
60
70
80
22-28
APase II
NaCl gradient, 0-500 mM
0
100
200
300
400
500
600
NaCl , mM
0
5
10
15
20
25
30
●——● , Protein conc., μg/0·3 ml

remained unadsorbned on rechromatography on a second DEAE - Sephadex column (Fig 4.13).

Sixty percent of the APase activity that was adsorbed was eluted with a gradient at about 78 mM NaCl, whereas most of the protein was eluted either in the run through fractions or at 98 mM and above NaCl. The purification fold and percent yield on the basis of whole microsomes were 39.2 and 16.7 respectively. The actual yield and purification would be greater than the observed value as the starting crude microsomes contain an unknown amount of other non-specific phosphatases.

The final step of purification was the ion-exchange chromatography on CM-Sephadex. The enzyme fractions eluted from DEAE-Sephadex column were dialyzed in 25 mM sodium acetate buffer pH 5.0, and applied after centrifugation to the CM-Sephadex column from which the enzyme was eluted with a linear 0 - 500 mM NaCl gradient. About 35% of inactive protein was precipitated during dialysis and 91% APase activity remained in solution. The overall purification and yield at this stage relative to the crude microsomes were 54.6 and 15.3 respectively. The elution profiles of the APase and protein are shown in Fig 4.14. A large amount of inactive protein was eluted in the void volume during the washing with the equilibration buffer. The total enzyme activity was, however, eluted in a sharp peak, fractions 22 to 28, at 100 mM NaCl gradient whereas a large protein peak with little or no APase activity was eluted at 350 mM NaCl gradient. The purification and yeild of APase were 95.8 fold and 1.1% respectively.

4.7 Homogeneity

On SDS-PAGE in the presence of 2-mercaptoethanol, the purified enzyme from the CM-Sephadex column gave a single protein band (Fig 4.15) corresponding to an apparent molecular weight of 68.0 kD (Fig 4.16). In order to determine that the protein band is actually due to APase, the native PAGE, (PAGE without SDS) was carried out at 4^{o}C under nondenaturation conditions. The gel was cut into 2 mm slices and each slice was individually homogenized in 1 ml of 50 mM sodium acetate buffer, pH 5.0, containing 100 mM NaCl and 0.1% CHAPS and centrifuged at 10,000 xg for 10 min. The supernatant was assayed for APase activity. The native PAGE showed a single protein band that coincided with the enzyme activity (Fig 4.17). These results confirm the purity of the enzyme preparation.

4.8 Characterization of the PM-APase

4.8.1 Stability

The purified enzyme was highly unstable and lost its total activity within 72 hours at 0^{o}C. However, at -20^{o}C the enzyme was stable for more than a week. This was in contrast to the PM-bound enzyme which could be stored at 0 - 4^{o}C for a month without any significant loss of activity.

4.8.2 Molecular size

The molecular mass of the native enzyme is in the range of 69,000 - 70,000 daltons as determined by comparison of the

FIG 4.15 : **Sodium dodecyl sulfate polyacrylamide gel electrophoresis of samples from various stages of purification of PM-APase from pea cotyledons**

Samples were prepared and handled as described under `Method´. Samples and amounts loaded were : PM, plasma membrane fraction, 100 µg protein, S.E., solubilized enzyme (dialyzed 105,000 xg supernatant), 80 µg protein; E.P, purified APase II enzyme, 50 µg protein; and St.P, standard molecular weight proteins: phosphorylase b, M.W. 92.3 kD; bovine serum albumin, M.W. 66.2 kD; ovalbumin, M.W. 45 kD; carbonic anhydrase, M.W. 31 kD; soybean trypsin inhibitor, M.W. 21.5 kD; and lysozyme, M.W. 14.4 kD; 5 µg each. Direction of electrophoresis was from top to bottom. The purified enzyme appeared as one protein band corresponding to a molecular weight of 68.0 kD.

FIG 4.15 : **Sodium dodecyl sulfate polyacrylamide gel electrophoresis of samples from various stages of purification of PM-APase from pea cotyledons**

Samples were prepared and handled as described under `Method´. Samples and amounts loaded were : PM, plasma membrane fraction, 100 µg protein, S.E., solubilized enzyme (dialyzed 105,000 xg supernatant), 80 µg protein; E.P, purified APase II enzyme, 50 µg protein; and St.P, standard molecular weight proteins: phosphorylase b, M.W. 92.3 kD; bovine serum albumin, M.W. 66.2 kD; ovalbumin, M.W. 45 kD; carbonic anhydrase, M.W. 31 kD; soybean trypsin inhibitor, M.W. 21.5 kD; and lysozyme, M.W. 14.4 kD; 5 µg each. Direction of electrophoresis was from top to bottom. The purified enzyme appeared as one protein band corresponding to a molecular weight of 68.0 kD.

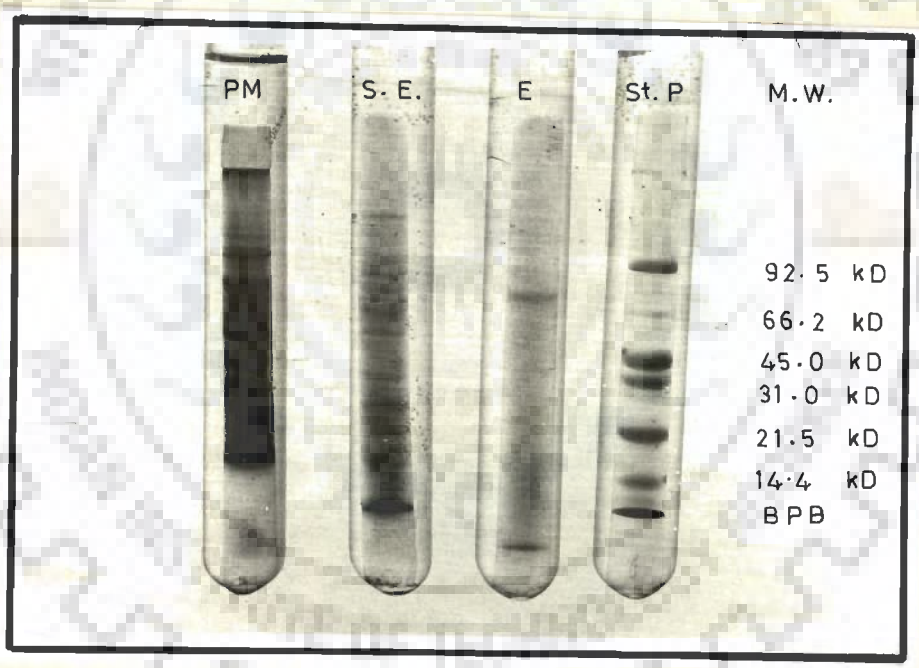

PM
S. E.
E
St. P
M.W.
92.5 kD
66.2 kD
45.0 kD
31.0 kD
21.5 kD
14.4 kD
BPB

FIG 4.16 : Molecular weight determination of the purified pea cotyledon plasma membrane acid phosphatase by SDS-PAGE

The standard proteins used were treated in the same manner as APase. Electrophoresis was performed on 10% polyacrylamide gel under fully dissociating conditions as described in `Methods´. Mobilities were determined relative to the migration of bromophenol blue as the tracing dye. Molecular weight standard proteins were : phosphorylase b (M.W. 92.5 kD); bovine serum albumin (M.W. 66.2 kD); ovalbumin (M.W. 45 kD); carbonic anhydrase (M.W. 31 kD); soybean trypsin inhibitor (M.W. 21.5kD) and lysozyme (M.W. 14.4kD). The molecular weight of the purified PM-APase as computed from the calibration plot of $\log_{10}$ molecular weight versus relative mobility of standard proteins, was found to be 68,000 daltons. The calculations were based on average of three independent electrophoretic runs.

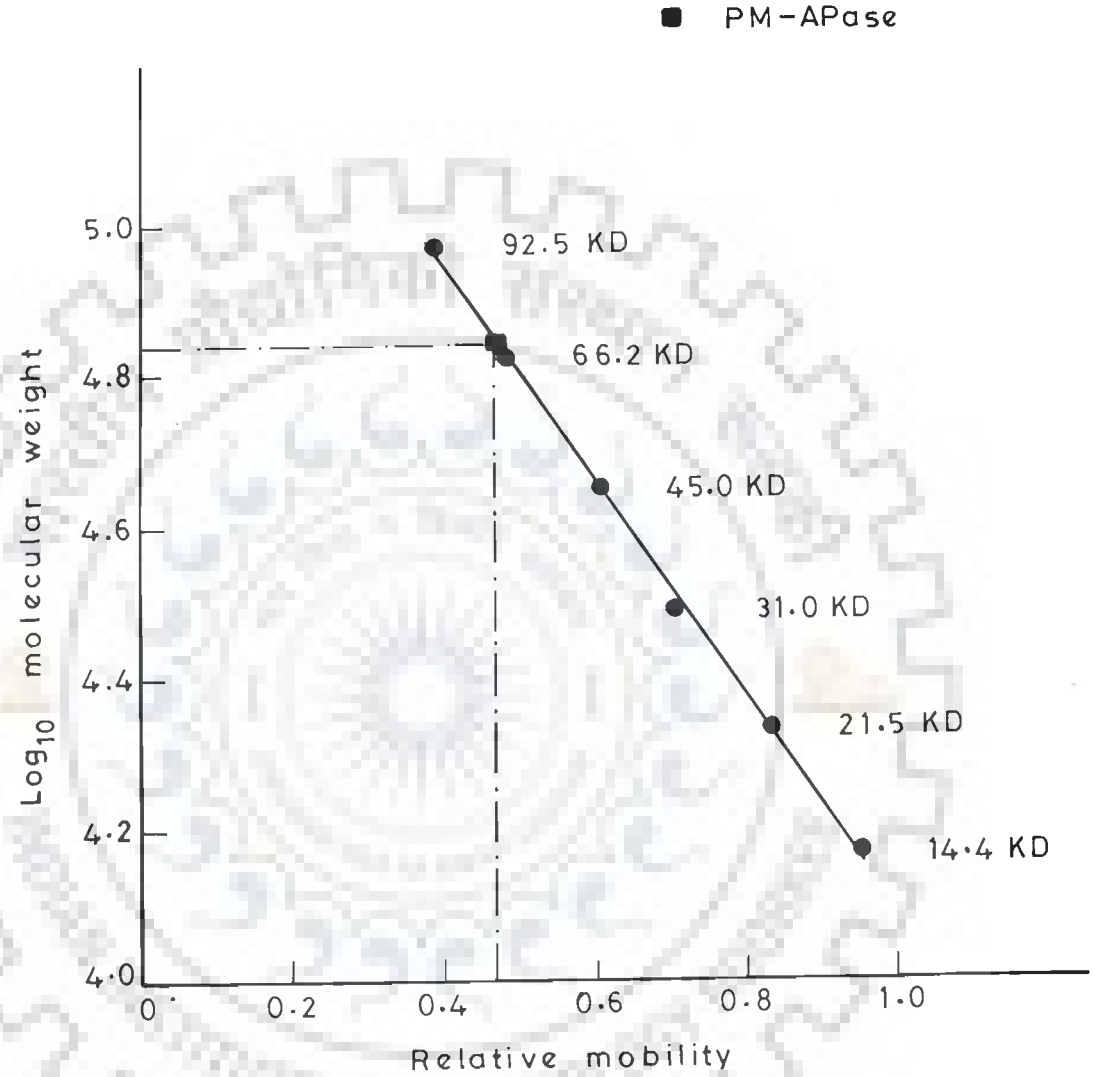
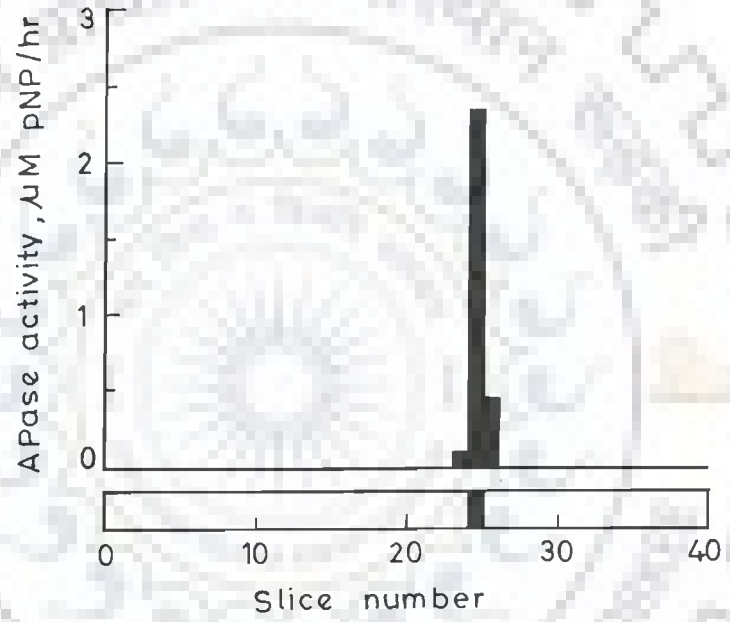


FIG 4.17 : Native gel (without SDS) of purified PM-APase

PAGE was carried out without SDS at 4°C under nondenaturation conditions. 2 mm slices were cut, homogenized in 1 ml 50 mM sodium acetate buffer, pH 5.0, containing 100 mM NaCl and 0.1% CHAPS, and centrifuged at 10,000 xg for 10 min. The supernatant was assayed for APase as described in `Methods´.


ATPase activity, μM pNP/hr
3
2
1
0
0
10
20
30
40
Slice number

elution position of APase with the elution position of standard proteins (Fig 4.18). The molecular mass of the enzyme as determined by the SDS/10% PAGE under reducing conditions yielded a value of 68,000 daltons (Fig 4.16) which is in close agreement with the value obtained by the gel filtration. These results indicate that the plasma membrane APase is a single polypeptide. It also rules out the possibility of aggregation during the gel filtration.

4.8.3 pH-Dependence and pH-stability

The APase from the pea cotyledons plasma membrane displayed maximal activity between the pH range 5.2 to 5.6. The enzyme activity below pH 5.0 and above pH 5.6 declines sharply (Fig 4.19). The enzyme was found to exhibit maximal stability over a broad pH range of 4.8 to 6.4 (Fig 4.19).

4.8.4 Kinetic properties

The Km and Vmax values for pNPP as substrate, as determined by Lineweaver-Burk plot (Fig 4.20), were found to be 3.1×10^{-4} M and 2 mM/min/mg protein, respectively. Iorganic phosphate (Pi) inhibited the enzyme in a competitive manner (Fig 4.20 and Fig 4.21a). The Ki value, as determined by replots of Lineweaver-Burk data (Fig 4.21 b,c) and Dixon plots (Fig 4.22), was found to be 0.4 mM. The Dixon plots intersect above the X-axis indicating further the competitive type of inhibition by Pi. Flouride inhibited the enzyme noncompetitively (Fig 4.20).

FIG 4.18 : Molecular weight determination by gel filtration on Sephadex G-200 (1.5 x 65 cm) of PM-APase

Inset shows the standard curve constructed from the elution volumes of marker proteins : 1, phosphorylase b (97,400 D); 2, BSA (66,000D);3, ovalbumin (42,700 D) and 4, cytochrome C (12,200 D). V_o and V_e are the void and elution volumes, respectively. V_o was the elution volume for blue dextran (average M.W. 2 x 10^6).

The column was run as described under `Methods´.

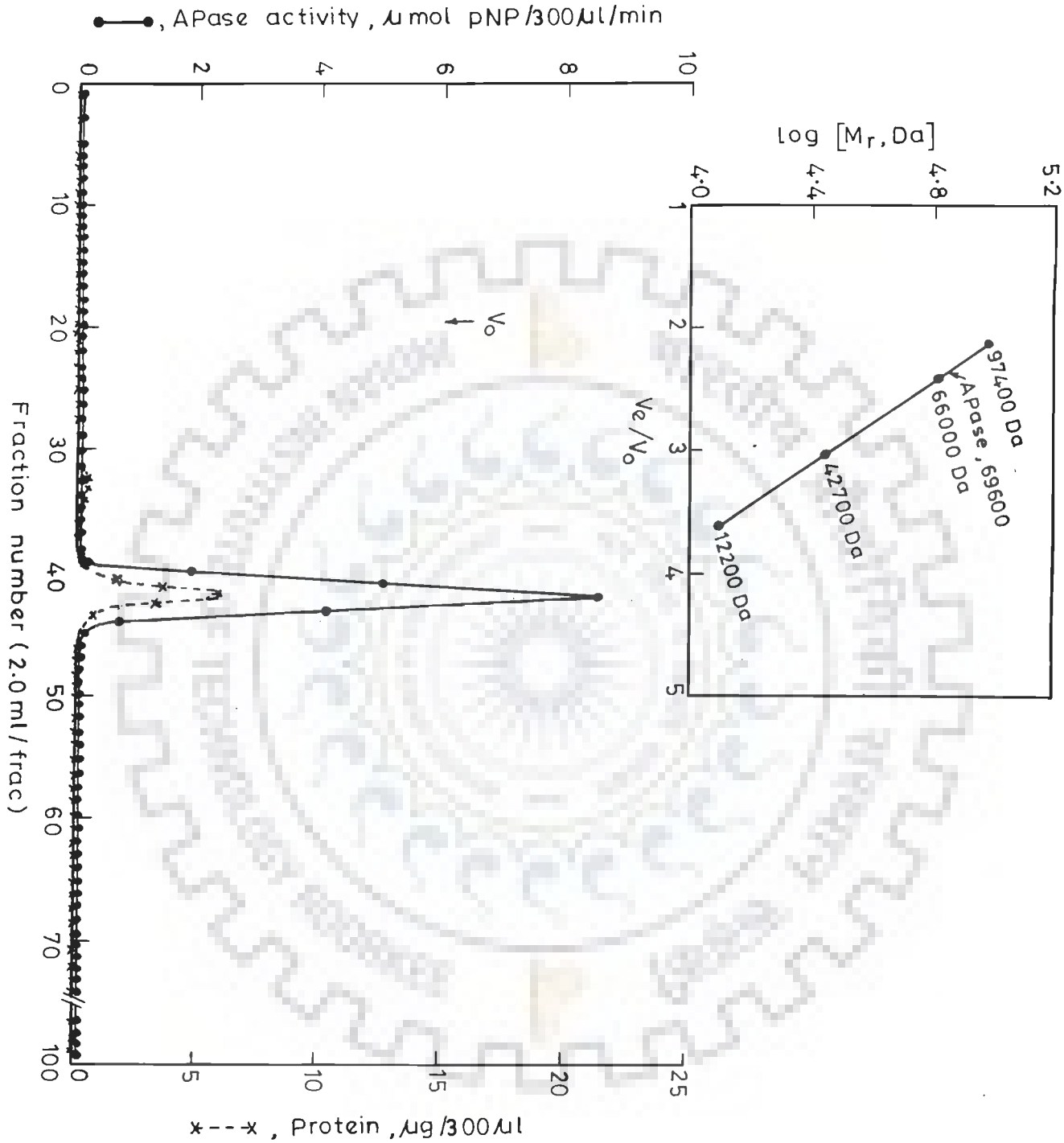

●——●, APase activity, μmol pNP/300μl/min
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80
90
100
Fraction number (2.0 ml/frac)
Vo
---, Protein, μg/300μl
0
5
10
15
20
25
log [Mr, Da]
4·0
4·4
4·8
5·2
1
2
3
4
5
Ve/Vo
97400 Da
APase, 69600
66000 Da
42700 Da
12200 Da

FIG 4.19 : pH-Optimum and pH-stability curves of PM-APase

The enzyme activity was measured by the standard assay system except that the buffer and pH varied. The buffers used were 50 mM acetate buffer pH 3.6-6.4, Tris-HCl, 50 mM, pH 6.8-8.

pH-Stability was determined by incubating the enzyme in the buffer of the indicated pH for 3h in ice and the remaining activity was assayed as usual.

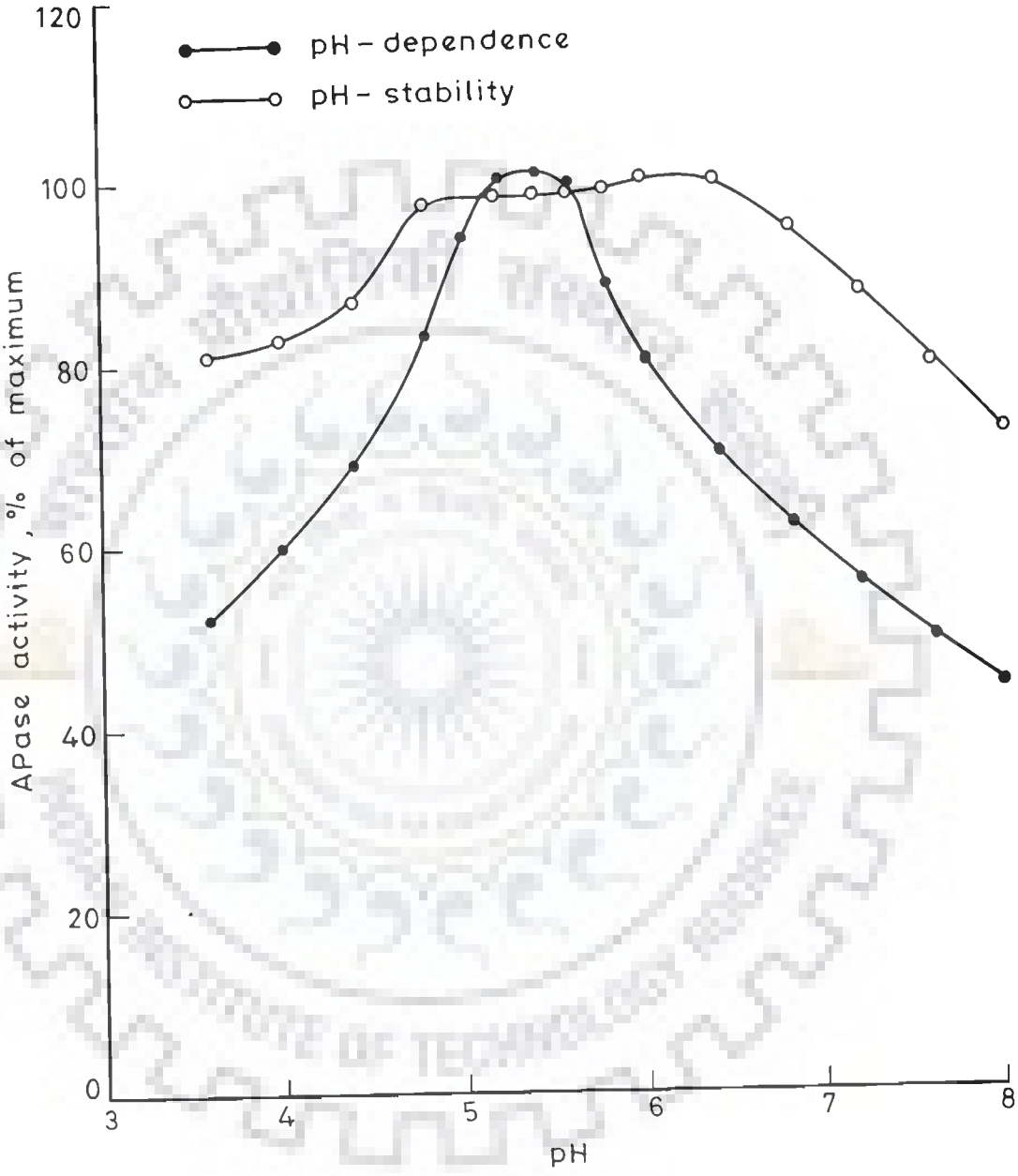

pH - dependence
pH - stability
ATPase activity, % of maximum
120
100
80
60
40
20
0
3
4
5
6
7
8
pH

FIG 4.20 : **Lineweaver-Burk plot of I/v versus I/[S] of PM-APase from pea cotyledons**

Standard assays using varying amounts of pNPP as substrate were carried out for 30 min with 10 ug enzyme. Km and Vmax values were computed from the intercepts on X-axis and Y-axis respectively.

The mode of inhibition by Pi and F^-, was determined by adding 0.5 mM (final concentration) inhibitor to the reaction mixture of each substrate concentration and assayed by the routine method. Pi and F^- inhibited the enzyme competitively and noncompetitively, respectively.

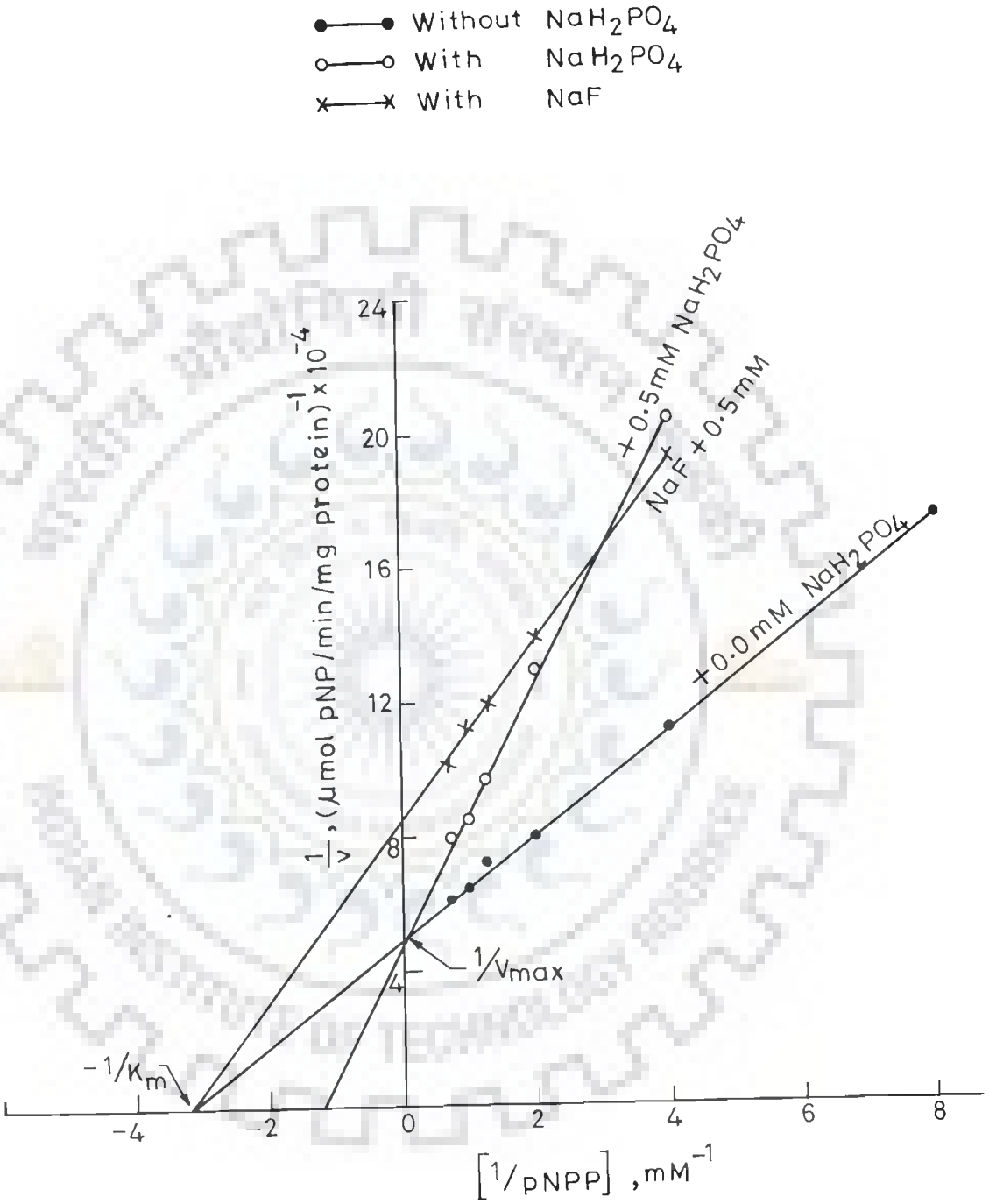

Without NaH_2PO_4
With NaH_2PO_4
With NaF
+0.5mM NaH_2PO_4
NaF +0.5mM
+0.0mM NaH_2PO_4
$\frac{1}{v}$, (µmol pNP/min/mg protein)$^{-1}$ x 10^{-4}
24
20
16
12
8
4
$1/V_{max}$
$-1/K_m$
-4
-2
0
2
4
6
8
$[1/pNPP]$, mM^{-1}

FIG 4.21 : Inhibition of pNPP hydrolysis by NaH_2PO_4

Enzyme assays were carried out using varying amounts of pNPP in the presence of various concentrations of NaH_2PO_4 (0.25, 0.5, 0.75 and 1.0 mM).

(A) Lineweaver-Burk plot of I/[S] verus I/v showing competitive type of inhibition.

(B) Replot of apparent Km verus NaH_2PO_4 concentration. Intercept on X-axis equals the value of -Ki. Ki is equal to 0.4 mM.

(C) Replot of slope (Km/Vmax) versus NaH_2PO_4 concentration. The intercept on the X-axis equals the value of -Ki. Ki is equal to 0.4 mM.

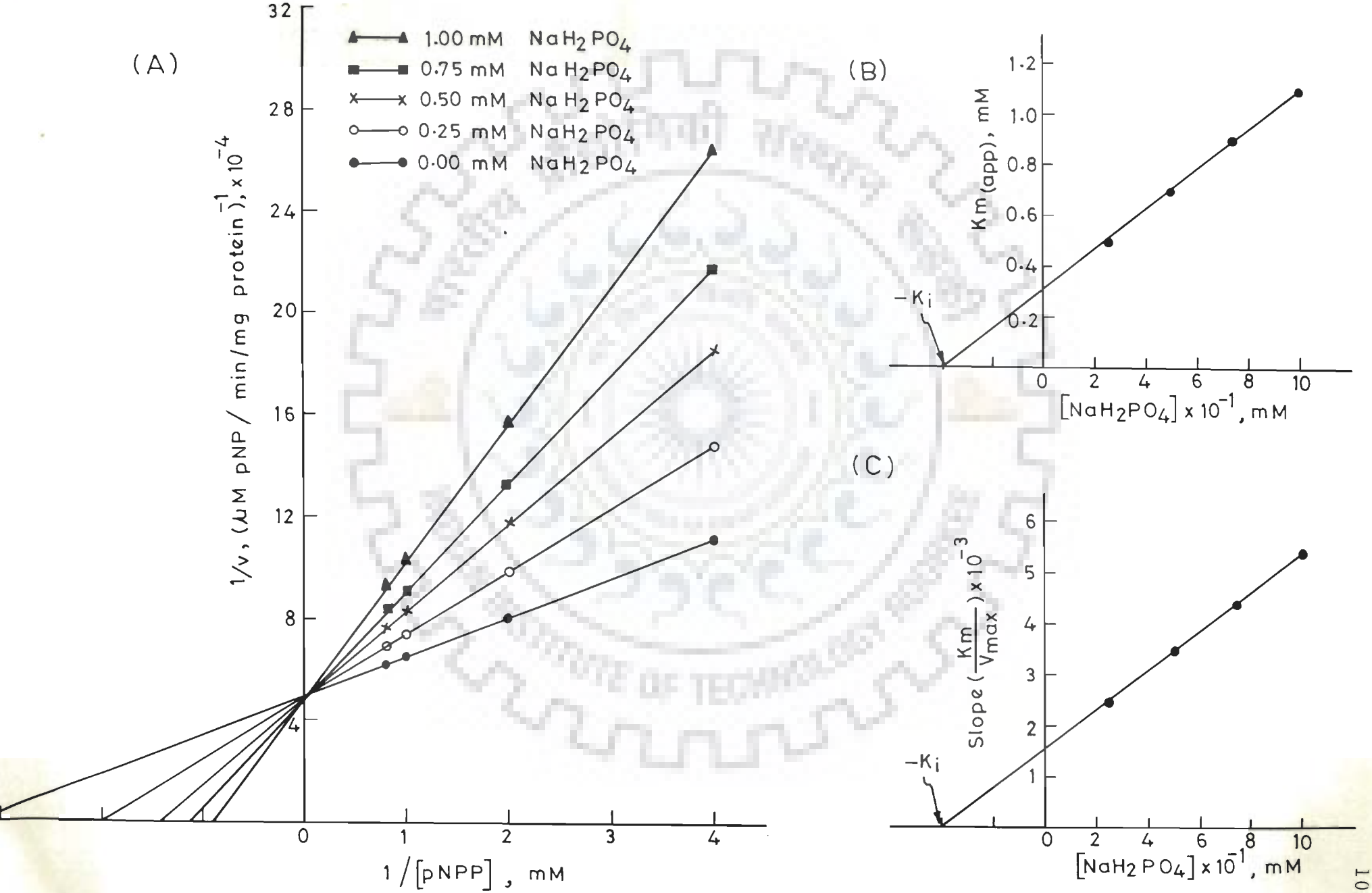
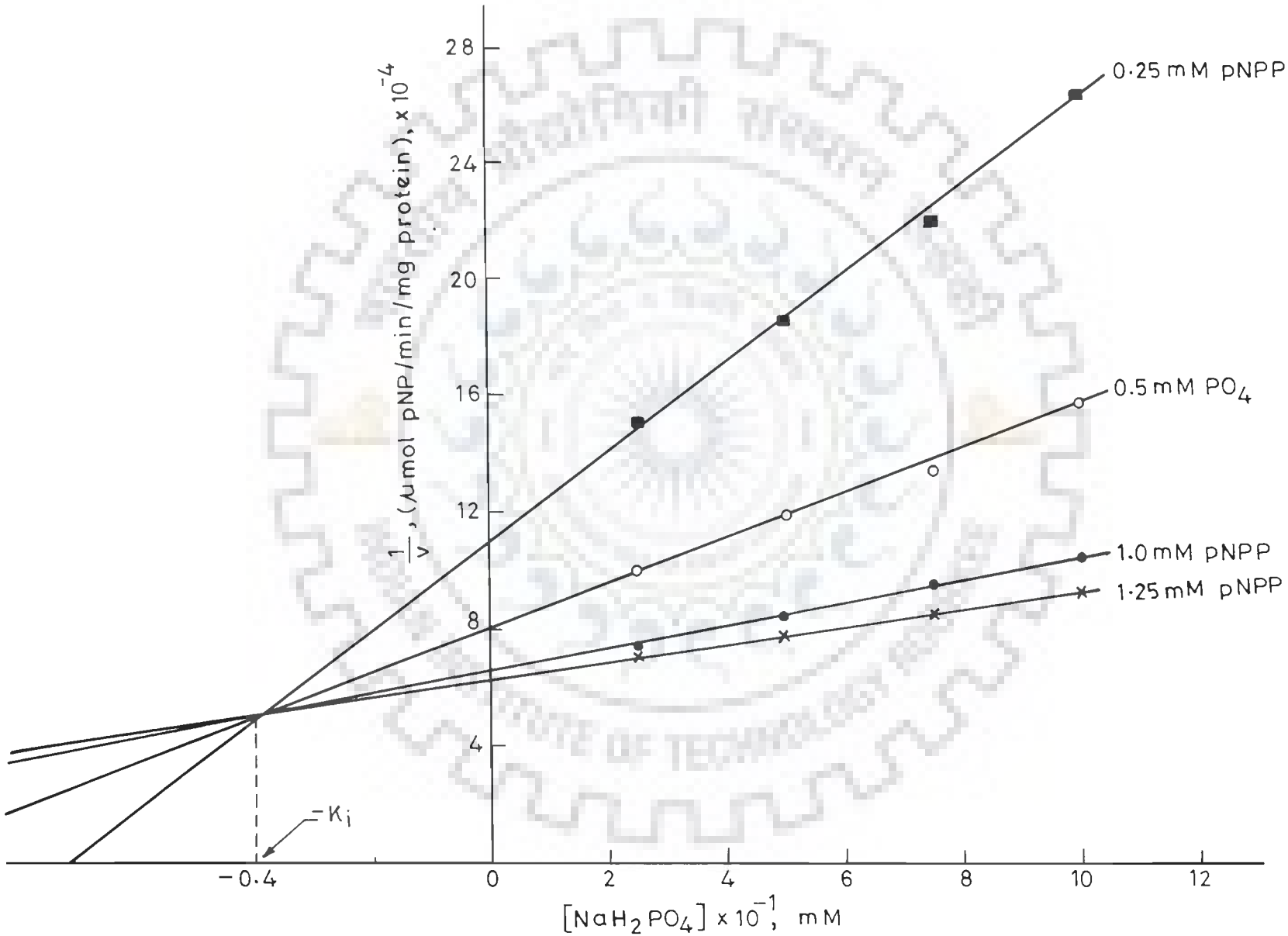

(A)
1.00 mM NaH2PO4
0.75 mM NaH2PO4
0.50 mM NaH2PO4
0·25 mM NaH2PO4
0·00 mM NaH2PO4
1/v, (μM pNP / min/mg protein)^-1, x 10^-4
1 / [pNPP], mM
(B)
Km(app), mM
-Ki
[NaH2PO4] x 10^-1, mM
(C)
Slope (Km/Vmax) x 10^-3
-Ki
[NaH2PO4] x 10^-1, mM

FIG 4.22 : Dixon plot of I/v versus NaH_2PO_4 concentration

Plots intersect above the X-axis indicating competitive type of inhibition. Ki is equal to 0.4 mM.


28
24
20
16
12
8
4
$\frac{1}{v}$, (µmol pNP/min/mg protein), x 10^{-4}
0·25 mM pNPP
0·5 mM PO_4
1·0 mM pNPP
1·25 mM pNPP
$-K_i$
−0·4
0
2
4
6
8
10
12
$[NaH_2PO_4] \times 10^{-1}$, mM

4.8.5 Substrate specificity

The substrates tested for the hydrolysis by the purified pM-APase from pea cotyledons are listed in Table XII. The enzyme was most active against p-nitrophenyl phosphate followed by ADP, CTP, GTP, ATP, UTP, GDP and UDP with relative rate of hydrolysis 100, 41.6, 37.5, 35.0, 33.5, 30.0, 29.3 and 29.3 percent, respectively. The nucleoside monophosphates namely AMP, CMP, UMP, GMP and phosphorylated sugars (glucose-6-phosphate, glucose-1-phosphate and glycerdehyde-3-phosphate) were not hydrolysed. Since the enzyme was found to be homogeneous by SDS-polyacrylamide gel electrophoresis, it appears unlikely that the hydrolysis of nucleoside di and triphosphates was due to contamination of acid phosphatases. The data, however, clearly show the non-specific nature of the PM-APase. The physiological significance of the non-specific property of the PM-APase is not clear.

4.8.6 Effect of some cations and anions

Table XIII shows the effect of some common metal ions on the purified PM-APase. The activity of the purified APase, under the assay conditions, was unaffected by the following cations (as chlorides) and anions at 10 mM final concentration : Na^+, K^+, citrate and tartarate. It may be pointed out here that citrate and tartarate were found to be strong activators of the PM associated APase. In contrast, there is no effect on the purified enzyme. Whether some protein is involved in the citrate

TABLE XII : Substrate specificity of the APase purified from the plasma membranes of pea cotyledons

The enzyme preparation (0.2 ml) was incubated with 1.0 mM substrate in 50 mM acetate buffer, pH 5.0 in a final volume of 1.5 ml. After 30 min incubation at 30°C, the reaction was terminated with 0.5 ml of 20% cold trichloroacetic acid. The denatured proteins (if any) were removed by centrifugation and Pi released was determined in the supernatant by the method of Fiske-SubbaRow (82). The relative rates of hydrolysis were determined relative to the pNPP which was taken as 100 percent.

Substrate	Activity of PM-APase μM Pi/min/mg protein	Relative to pNPP (percent)
pNPP	946.0	100.0
ATP	317.0	33.5
GTP	331.0	35.0
CTP	356.3	37.7
UTP	284.0	30.0
ADP	395.6	41.6
GDP	277.0	29.3
UDP	277.0	29.3
AMP	u.d.[a]	0.0
CMP	u.d.	0.0
GMP	u.d.	0.0
UMP	u.d.	0.0
Glc-6-p	u.d.	0.0
Glc-1-p	u.d.	0.0
Gly-3-p	u.d.	0.0

a u.d. undetectable

TABLE XIII : Effect of some common metal ions and anions on the activity of the APase from the plasma membranes of pea cotyledons

The enzyme was assayed in absence and presence of cations (as chlorides) and anions at a final concentration of 10 mM by the standard assay procedure. The relative rate of hydrolysis of pNPP in the presence of ions was determined relative to control which was taken as 100 percent. Controls were without additions.

Addition	Activity of APase μMpNP/min/mg protein	Percent of control %
None	1431.0	100.0
cations		
Mg^{2+}	1171.0	81.8
Ca^{2+}	1171.0	81.8
Zn^{2+}	43.0	3.0
Hg^{2+}	70.2	4.9
Cu^{2+}	43.0	3.0
Mn^{2+}	472.5	33.0
Ni^{2+}	858.6	60.0
K^{+}	1432.0	100.0
Na^{2+}	1432.0	100.0
Anions		
F^{-}		
PO_4^{3-}		
$Mo_7O_{24}^{6-}$		
Citrate	1447.2	101.0
Tartarate	1458.0	101.8

and tartarate activation of the PM-bound enzyme, which could have been removed during the purification of the APase is not known. Lack of effect by Na^+ or K^+ shows the absence of Na^+ or K^+ activated ATPases. Mg^{2+} and Ca^+ show slight inhibition (ca 18%). There results also indicate the absence of Mg^{2+} or Ca^{2+} - dependent ATPases. The hydrolysis of ATP by the purified APase, which is about 35% of the hydrolysis rate of pNPP (Table XII) may be attributed to the non-specific action of the APase.

Zn^{2+}, Hg^{2+} and Cu^{2+} ions are strong inhibitors, inhibiting almost the whole of enzyme activity. Mn^{2+} and Ni^{2+} were also found to inhibit the purified enzyme to about 33 and 60%, repsecitvely. The strong inhibition of APase by Hg^{2+}, Zn^{2+} and Cu^{2+} suggests that SH group of cystein residues may be involved in the interaction between the substrate and the enzyme. As expected, the purified APase was also strongly inhibited by fluoride, Pi and molybdate.

4.8.7 Glycoprotein nature of PM-APase

The pea cotyledon PM-APase was found to contain 21.1% carbohydrate and appears to be a glycoprotein. After reducive SDS-PAGE, the single APase band gave a positive reaction for periodate/Schiff base staining for carbohydrates (279). Digestion of the enzyme with endo—N-acetyl — β-D-glucosaminidase H (endo-H) released about 70% of the total carbohydrate from the enzyme (Fig 4.23), indicating that the oligosaccharide moiety was asparagine-linked high mannose type.

FIG 4.23 : Deglycosylation of pea cotyledon plasma membrane APase by endo-N-acetyl-β-D-glucosaminidase H (endo-H)

The purified enzyme was incubated with endo-H enzyme at a concentration of 50 mU/ml for the indicated periods. The reaction was stopped by the addition of 3 volumes of ice cold ethanol. The sample was centrifuged at 10,000 xg for 15 min and the supernatant was saved. The pellet was washed 2x with 1 ml 75% ethanol and recentrifuged. The supernatant and washings were pooled and assayed for carbohydrate by the phenol-sulphuric acid method (71) as described under `Methods'. Controls were without endo-H.

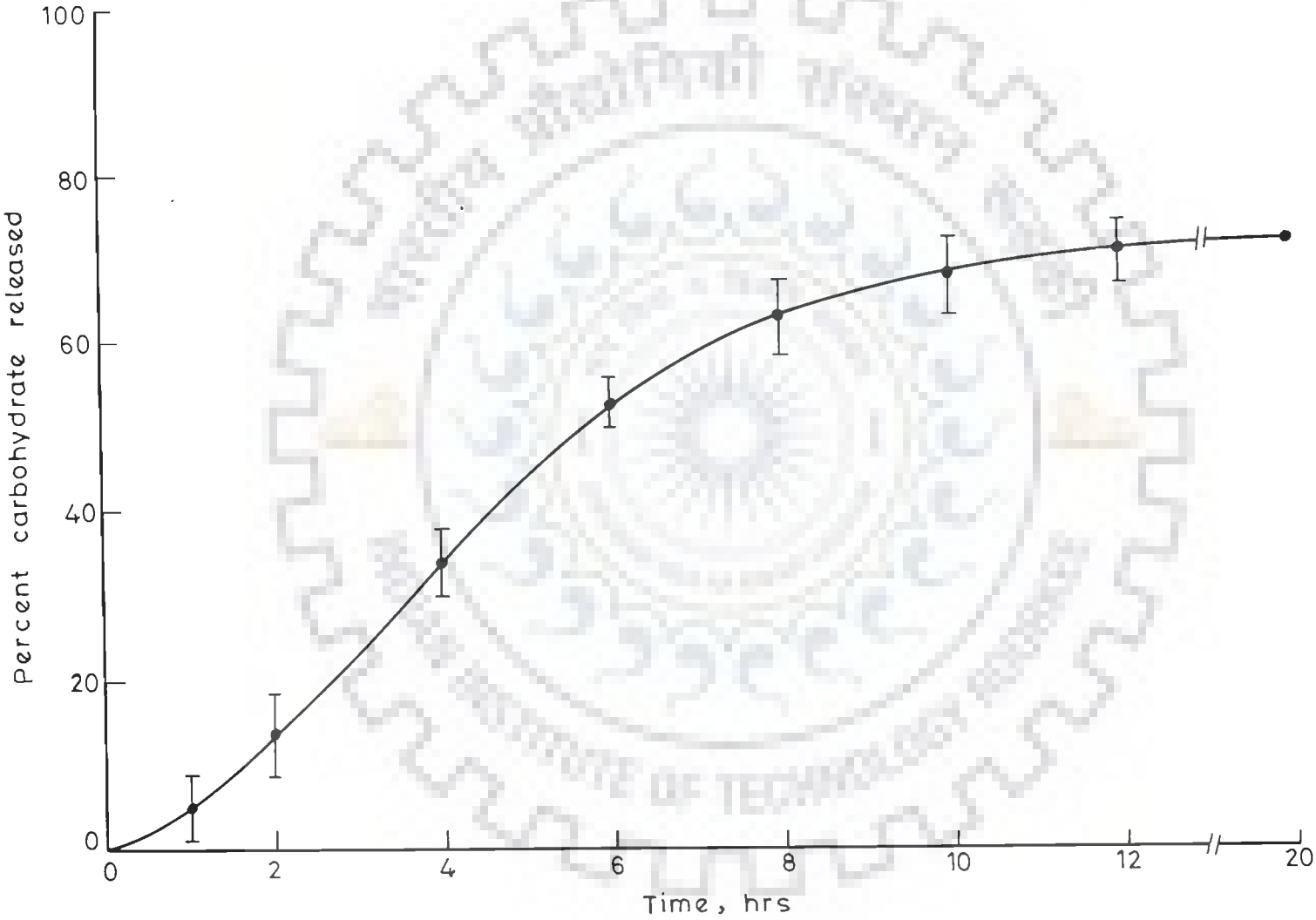
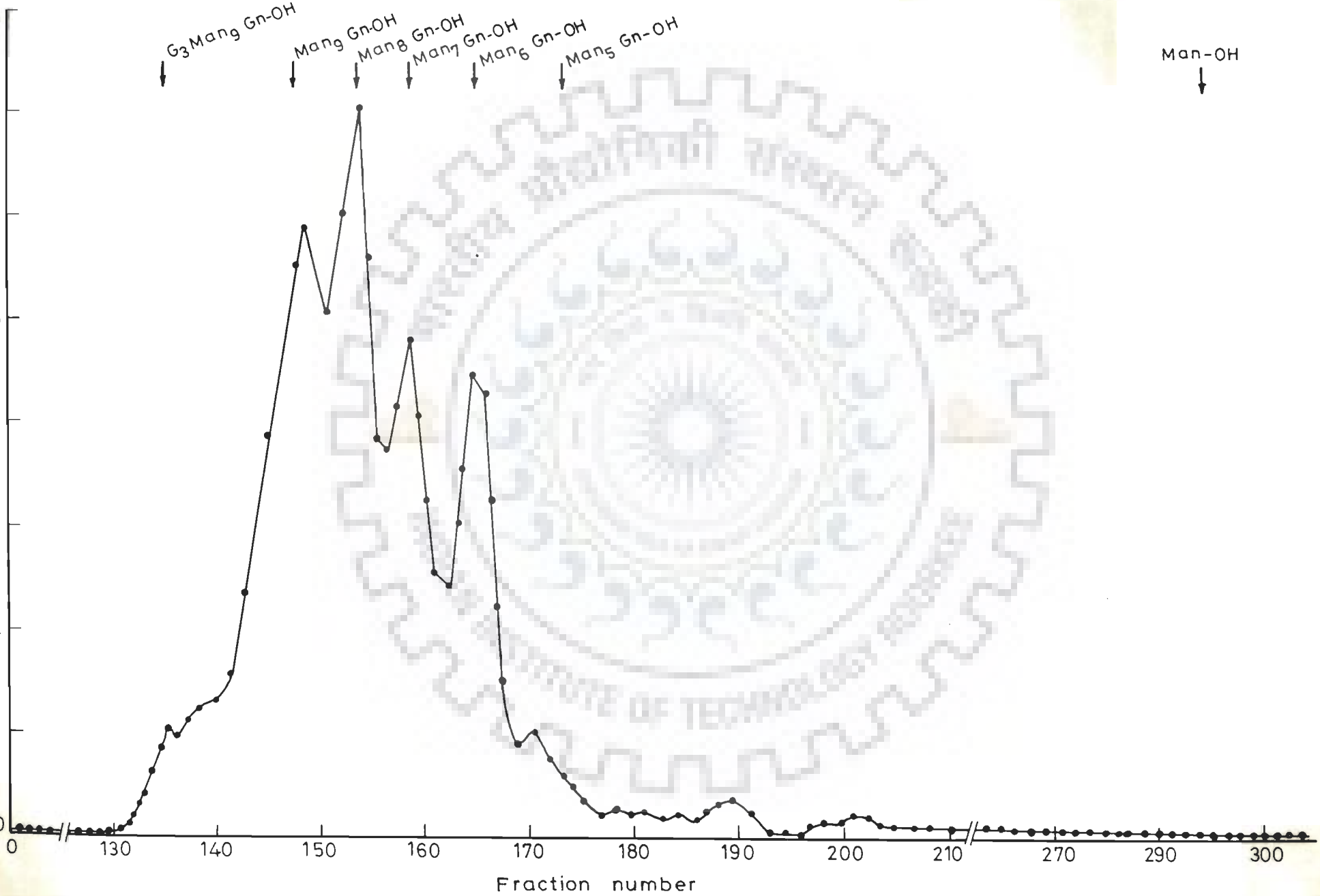

Percent carbohydrate released
100
80
60
40
20
0
0
2
4
6
8
10
12
20
Time, hrs

FIG 4.24 : Bio-Gel P-4 chromatography of oligosaccharides released from PM-APase by endo-H

The oligosaccharides were reduced with NaB^3H_4 and the reduced ^3H-labelled sugar alcohols (Man_x-GnO^3H) were resolved in Bio-Gel P-4 column (2 x 200 cm) using radioactive labelled mannitol and other oligosaccharides as standards. Elution was done with 0.01 M sodium acetate, pH 5.0 One ml fractions were collected at a flow rate of 6 ml/h and countred in a Beckman liquid scintillation counter. Arrows show the position of standards : G = glucose; Man = mannose; Gn-OH = N-acetylglucosaminitol.


G3Man9 Gn-OH
Man9 Gn-OH
Man8 Gn-OH
Man7 Gn-OH
Man6 Gn-OH
Man5 Gn-OH
Man-OH
0
130
140
150
160
170
180
190
200
210
270
290
300
Fraction number

About 30% of the carbohydrate moiety was not endo-H senstitive. At the moment the nature of the endo-H resistant oligosaccharide moiety is unknown.

The high mannose type oligosaccharides from APase were analyzed further on Bio-Gel P-4 column. Endo-H-released oligosaccharides were separated from the deglycosylated protein by precipitation with 3 volumes of ethanol followed by certrifugation at 10,000 xg for 15 min. The supernatant, containing oligosaccharides, was dried under reduced pressure and reduced with tritium-labelled sodium borohydride (NaB^3H_4) (11) . The radiolabelled saccharides were then separated by ion-exchange and paper chromatography. These steps removed almost all contaminating matter and were suitable for both Bio-Gel P-4 and HPLC analysis. Fig 4.24 shows the elution profile of the reduced highmannose oligosaccharides from the pea cotyledon plasma membrane APase. These results show the presence of five major radioactive peaks corresponding to hexamannosyl-N-acetylglucosaminitol (Man_6GnOH), heptamannosyl-N-acetylglucosaminitol (Man_7GnOH), octamannosyl-N-acetylglucosaminitol (Man_8GnOH) and nanomannosyl-N-acetylglucosaminitol (Man_9GnOH). The relative abundance of these oligosaccharides appears to be Man_8 GnOH, Man_9GnOH, Man_7GnOH and Man_6GnOH. However, further characterization of these oligosaccharides would be necessary.

4.8.8 Is carbohydrate moiety essential for APase Activity ?

In order to know whether or not the carbohydrate moiety is required for the activity of the enzyme, the purified enzyme was

TABLE XIV : Effect of periodate oxidation on the plasma membrane APase activity

The purified APase from the pea cotyledon plasma membranes was incubated without and with $NaIO_4$ (10 mM) in 50 mM sodium acetate buffer, pH 5.0, for indicated periods in the dark at 4°C, and the APase activity was assayed after the addition of ethylene glycol and dialysis against the incubation buffer.

Time of incubation (min)	APase activity[a]			
	+$NaIO_4$		-$NaIO_4$	
	µMpNP/min/mg protein x10^2, ±SD	%[b]	µMpNP/min/mg protein x10^2, ±SD	%
0	14.1 ± 1.3	100.0	14.1 ± 1.2	100.0
15	12.6 ± 1.3	89.3	14.2 ± 1.2	100.7
30	8.3 ± 1.0	58.8	13.8 ± 1.2	97.8
60	4.5 ± 0.5	31.9	13.9 ± 1.2	98.5
120	2.4 ± 0.3	17.0	14.3 ± 1.2	101.5

a The results are average of three experiments.

b Values are percent of controls ran concurrently.

TABLE XV : Pea cotyledon plasma membrane APase activity after deglycosylation with endo-N- β-D-acetylglucosaminidase H (endo-H)

The purified enzyme was incubated with endo-H at a concentration of 50 mU/ml at 37°C in 50 mM citrate buffer, pH 5.0, for the periods indicated and then assayed for the APase activity. Controls were without endo-H.

Period of incubation (hours)	APase activity (μMpNP/min/mg protein)[a], ±SD		
	Control	Endo-H treated	% Control
0	1040 ± 50	1040 ± 50	100.0
1	1012 ± 50	881 ± 45	87.0
2	989 ± 45	764 ± 45	77.2
4	943 ± 45	621 ± 40	65.8
8	872 ± 42	483 ± 40	55.4

a Average of three experiments.

treated with periodate for varying intervals of time and the enzyme activity was assayed. Table XIV summarizes the results of this experiment. It was noticed that treatment of the enzyme with $NaIO_4$ progressively decreases the activity of the APase. For instance, after 60 min incubation with $NaIO_4$ at 4^oC, about 70% activity of the enzyme was lost, whereas control incubations (without $NaIO_4$) showed no loss of enzyme activity. These results clearly suggest, but by no means prove, that carbohydrate moiety may be essential for the enzyme activity.

The change in the APase activity during the deglycosylation of the enzyme by endo-H was also studied. The results are shown in Table XV. The APase activity declines as the deglycosylation proceeds suggesting, once again, the essential role of carbohydrate moiety in the activity of APase.

4.9 Golgi apparatus acid phosphatase

4.9.1 Purification of Golgi APase

The GA-APase was purified using nearly the same purification scheme as was used for the purification of PM-APase. The only exception was that, DEAE-Sephadex step was replaced by the Sephadex G-100 gel filtration, since the former resulted in almost total inactivation of the GA-APase. The purification data are summarized in Table XVI. Separation of Golgi apparatus from the crude microsomal fraction itself yielded a 2-fold purification with the yield of 4.2 percent. Like the PM-APase, the GA-APase was efficiently solubilized by CHAPS. Under the

TABLE XVI : Purification of acid phosphatase from Golgi apparatus

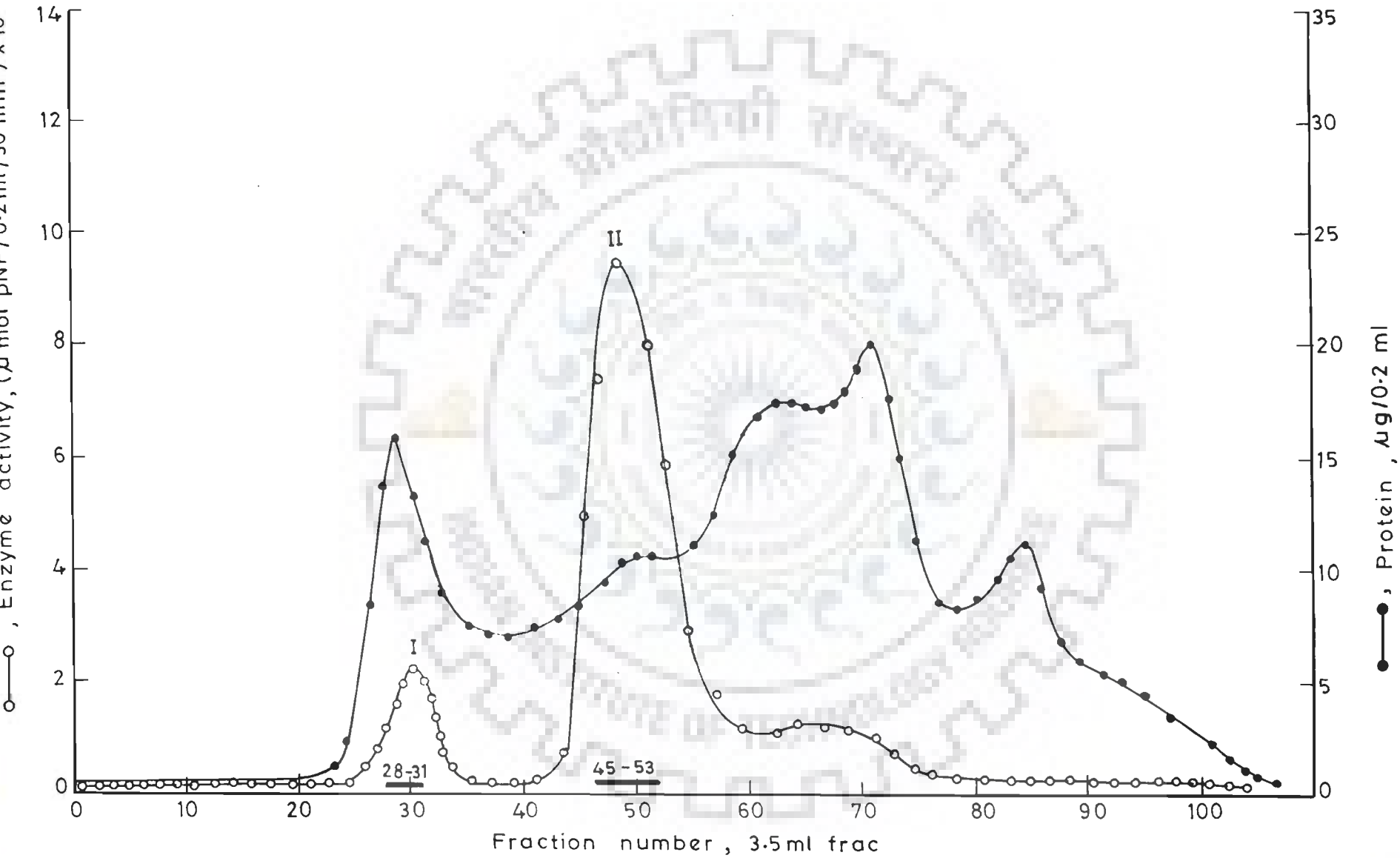
Highly enriched Golgi apparatus fraction from pea cotyledons, prepared as described in text, was used to purify APase using essentially the same purification scheme as described for the PM-APase purification.

Fraction	Total protein (mg)	Total APase activity µMpNP/min	Specific APase Activity µMpNP/min/ mg protein	Purification (fold)***	Yield %
Crude microsomes* (12,000-105,000 xg) pellet	1950.0	24433.0	12.53	1.00	100.0
Golgi apparatus	40.0	1030.0	25.75	2.05	4.20
CHAPS extract (105,000 xg supernatant)	11.4	761.0	66.77	5.33	3.10
Dialysis at pH 5.0** (clear supernatant)	6.5	621.0	95.47	7.62	2.50
Sephadex G-100	1.8	407.0	226.11	18.04	1.70
CM-Sephadex	0.26	178.0	684.80	54.65	0.73

* From three different batches of preparations.

** Dialysis at pH 5.0 for 12 h at 0-4°C resulted in precipitation of significant amount of inactive protein with very little APase activity (about 16 percent) which was removed by centrifugation and supernatant fraction was used.

*** Actual purification would be much higher than observed because of non-specific APases present in GA and instability of the purified enzyme.


14
12
10
8
6
4
2
0
35
30
25
20
15
10
5
0
I
II
28-31
45-53
0
10
20
30
40
50
60
70
80
90
100
Fraction number , 3.5ml frac
, Protein , µg/0·2 ml

same conditions used for the solubilization of the PM-APase, nearly three fourths activity was released from the GA-membranes in stable and active form. In addition, the specific activity of the CHAPS-solubilized enzyme was 2.6 times more than that of the GA, suggesting a kind of selective solubilization of the APase.

When the solubilized enzyme was dialyzed overnight against 25 mM sodium acetate buffer, pH 5.0, significant amount of inactive protein was precipitated and over 80% APase activity remained in solution. The overall purification and yield at this stage, relative to the crude microsomes, were 7.6-fold and 2.5% respectively. The next purification step was gel filtration on Sephadex G-100. The elution was performed with the equilibration buffer i.e. 25 mM sodium acetate, pH 5.0. The elution profile is shown in Fig 4.25. Two APase containing peaks (peak I, 28 - 31 fractions and peak II, 45 - 53 fractions) were obtained. Of these, peak II was predominant and accounted for most of the enzyme activity eluted with an overall purification of 18-fold and 1.7 percent yield. Upto this stage of purification the enzyme was fairly stable. Peak II was purified further on CM-Sephadex. The elution profiles of the enzyme and protein are shown in Fig 4.26. A substantial amount of protein along with a relatively small amount of APase activity was eluted in the void volume. However, a large amount of APase activity was adsorbed on the CM-Sephadex column and was eluted in a single sharp peak (fraction numbers 27 to 32) at 100 mM NaCl gradient. Top three fractions containing the enzyme activity were pooled. This step

FIG 4.26 : CM-Sephadex of GA-APase II (peak II from Sephadex G-100 gel filtration)

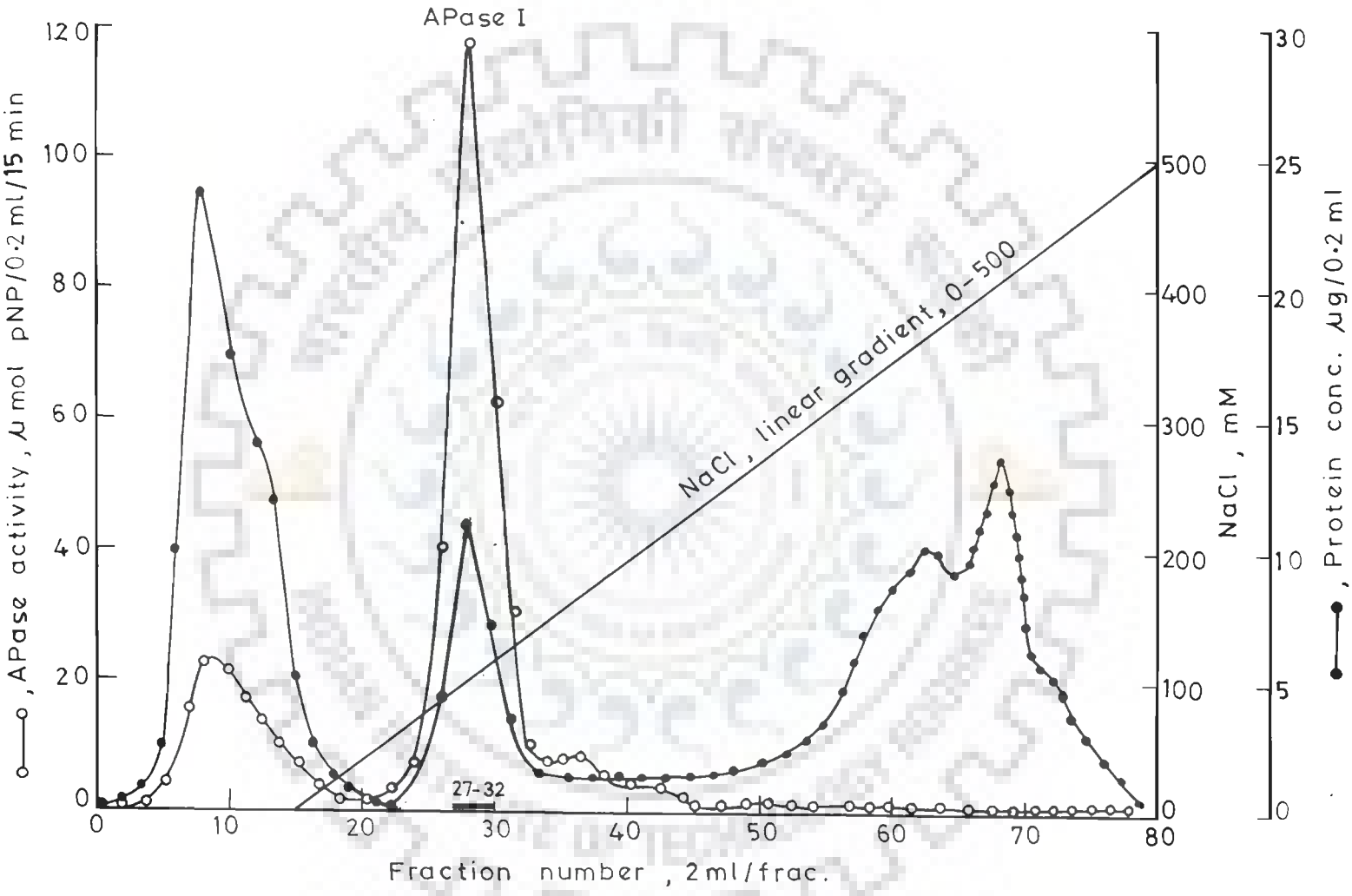
Experimental conditions were those used for the purification of the plasma membrane APase.

FIG 4.25 : Gel filtration on Sephadex G-100 of the dialyzed solubilized GA-APase

A 1.5 x 50 cm column pre-equilibrated with 25 mM sodium acetate buffer, pH 5.0, was used. 2 ml (6.5 mg) protein were loaded on the column and eluted with the equilibration buffer at a flow rate of 6 ml/h. 3.5 ml fractions were collected and an aliquot (0.2 ml) from every other tube was analyzed for the APase activity and protein concentration.

FIG 4.27 : Sodium dodecyl sulfate polyacrylamide gel electrophoresis of samples of various stages of purification of GA-APase from pea cotyledons

Experimental details were as those described in Fig 4.15.


APase I
120
100
80
60
40
20
0
o—, APase activity, μ mol pNP/0·2 ml/15 min
0
10
20
30
40
50
60
70
80
Fraction number, 2 ml/frac.
27-32
NaCl, linear gradient, 0–500
500
400
300
200
100
0
NaCl, mM
30
25
20
15
10
5
0
●—, Protein conc. μg/0·2 ml

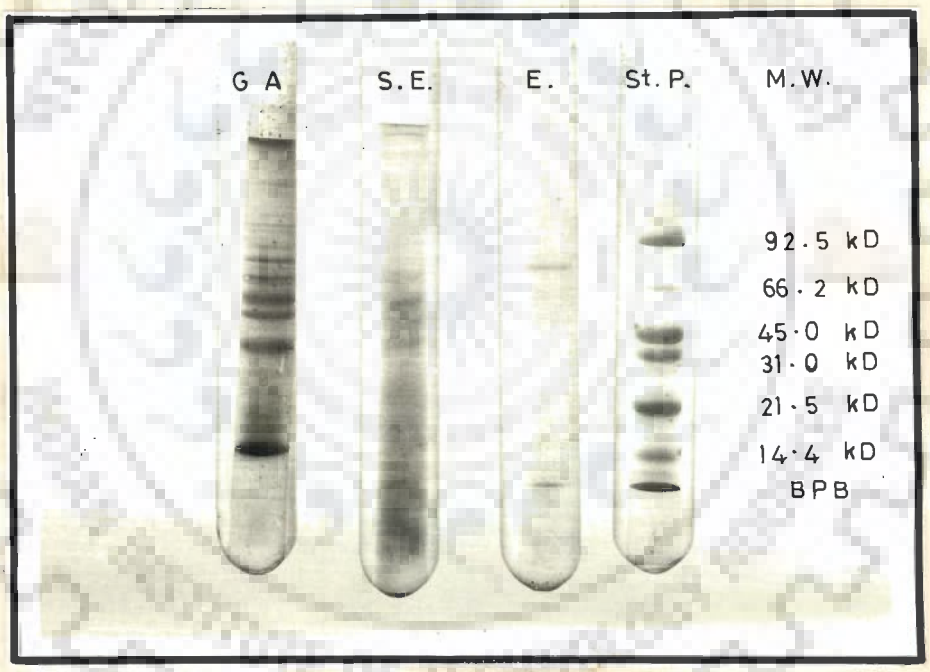

G A
S.E.
E.
St. P.
M.W.
92.5 kD
66.2 kD
45.0 kD
31.0 kD
21.5 kD
14.4 kD
BPB

FIG 4.28 : Molecular weight determination of the purified GA-APase from pea cotyledons by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

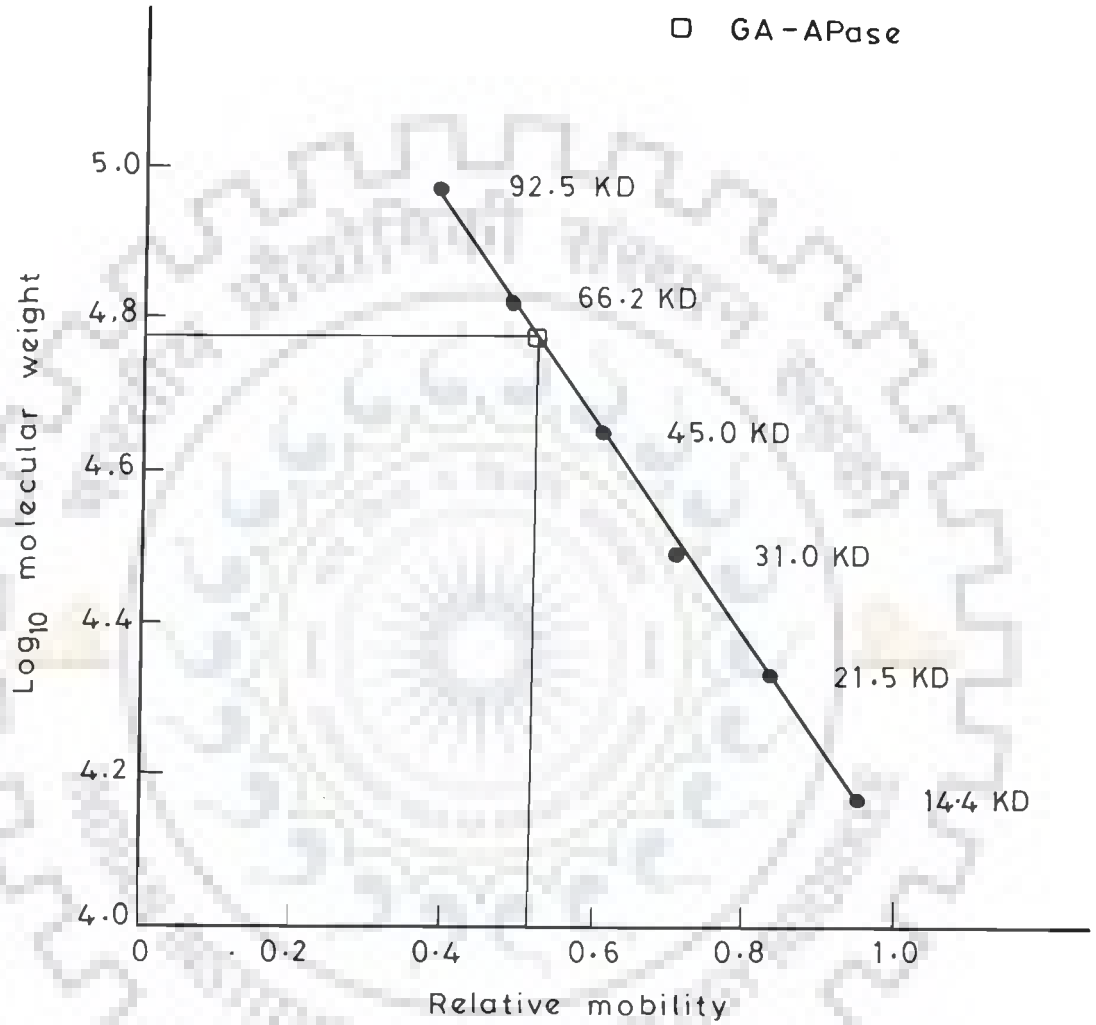
Experimental details were as those described for Fig 4.16 ran concurrently.

GA : Golgi apparatus

S.E. : CHAPS-solubilized GA-APase

E : Purified GA-APase

St. P. : Standard molecular weight proteins.


GA-APase
92.5 KD
66.2 KD
45.0 KD
31.0 KD
21.5 KD
14.4 KD
5.0
4.8
4.6
4.4
4.2
4.0
Log10 molecular weight
0
0·2
0·4
0·6
0·8
1.0
Relative mobility

resulted in a 3-fold purification over the gel filtration step. The final purification and yield with respect to the crude microsomal fraction were 54.65 fold and 0.73%, respectively. The enzyme preparation was found to be homogeneous by SDS-PAGE (Fig 4.27) with and apparent molecular weight of 66.1 kD. (Fig 4.28).

4.9.2 Properties of the GA-APase

4.9.2.1 Molecular weight

The molecular weight of the GA-APase, as determined by the gel filtration on Sephadex G-200 (Fig 4.29) was found to be 65.5 kD which is in fair agreement with that determined by SDS-PAGE (61.1 kD, Fig 4.28) indicating that the enzyme is probably composed of a single unit. It may also be pointed out that the enzyme was eluted in a single sharp peak which further suggested the homogeneity of the enzyme.

4.9.2.2 Kinetic properties

The enzyme showed a broad pH-optimum from pH 5.2 to 5.6 (Fig 4.30). The enzyme activity decreased rather sharply below or above the optimum pH. The values of apparent Km and Vmax as determined by the double reciprocal plots (Fig 4.31) using p-nitrophenyl phosphate as substrate were found to be 3.6×10^{-4} M and 0.87 mM/min/mg protein, respectively. F^- and PO_4^{3-} inhibited the enzyme noncompetitively and competitively, respectively. (Fig 4.31).

FIG 4.29 : **Molecular weight determination of GA-APase by gel filtration on Sephadex G-200**

Enzyme loaded was APase II purified on CM-Sephadex. Experimental conditions were those described in Fig 4.18. V_o = void volume; V_e = elution volume.

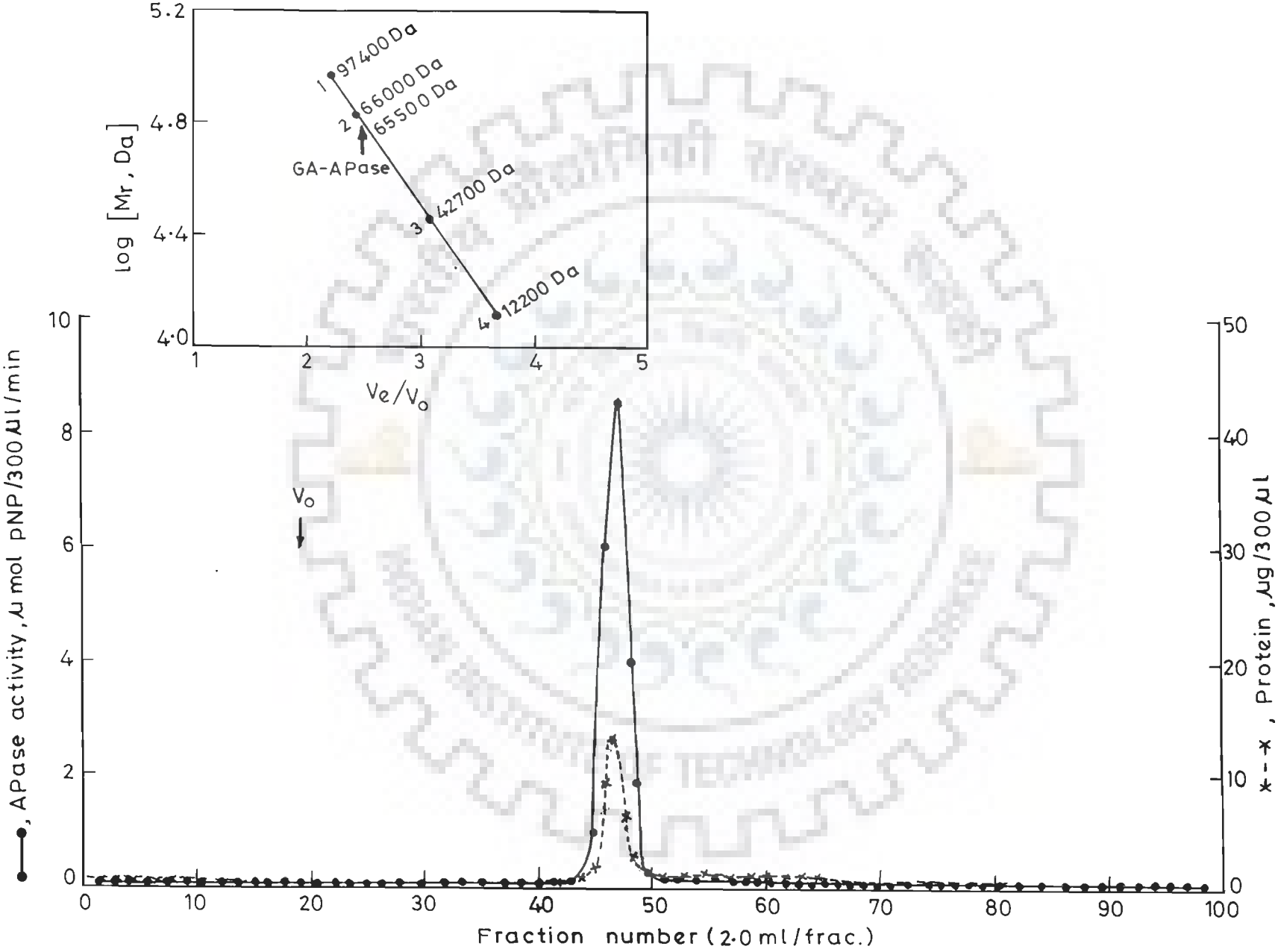

5.2
4.8
4.4
4.0
Log [Mr, Da]
1
97400 Da
2
66000 Da
65500 Da
GA-APase
3
42700 Da
4
12200 Da
1
2
3
4
5
Ve/Vo
Vo
10
8
6
4
2
0
●, APase activity, μ mol pNP/300 μl/min
50
40
30
20
10
0
-, Protein, μg/300 μl
0
10
20
30
40
50
60
70
80
90
100
Fraction number (2.0 ml/frac.)

FIG 4.30 : Optimum pH of the purified APase from the Golgi apparatus of pea cotyledons

The rate of hydrolysis of p-nitrophenyl phosphate by the purified enzyme was determined by the standard enzyme assay at varied pH. The buffers used were 50 mM sodium acetate, pH 3.6 to 6.4 and 50 mM Tris-HCl, pH 6.8 - 8.0.

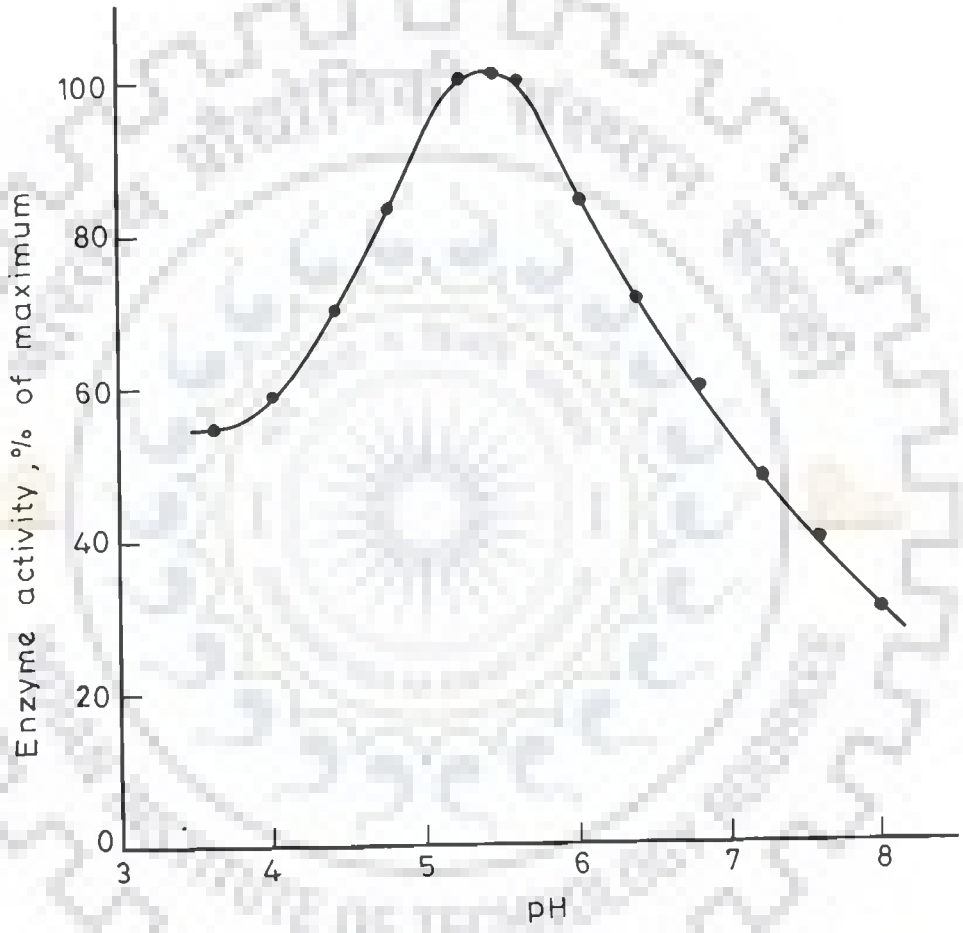

Enzyme activity, % of maximum
100
80
60
40
20
0
3
4
5
6
7
8
pH

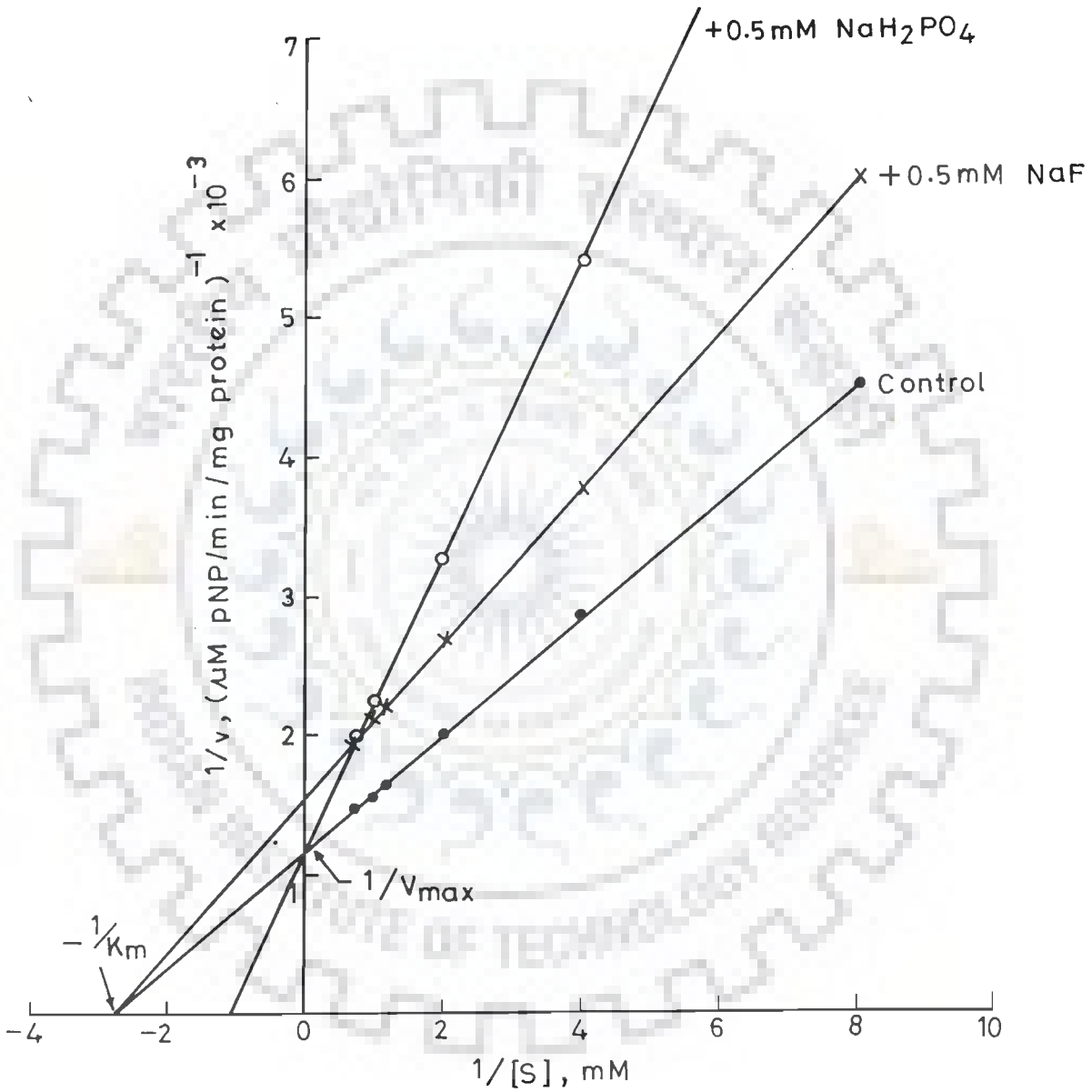
FIG 4.31 : Double reciprocal plot of the purified APase from the Golgi apparatus of pea cotyledons

Standard assays were carried out with varying amounts of p-nitrophenyl phosphate for 30 min with fixed amount of enzyme. Km and Vmax were computed from the intercepts of X-axis and Y-axis, respectively.

Mode of inhibition by inorganic phosphate and F^- were competetive and noncompetitive, respectively.

Km = 3.6×10^{-4} mM

Vmax = 0.87 mMpNP/min/mg protein


+0.5mM NaH2PO4
x +0.5mM NaF
Control
1/v, (μM pNP/min/mg protein)^-1 x10^-3
1/Vmax
-1/Km
1/[S], mM
7
6
5
4
3
2
1
-4
-2
0
2
4
6
8
10

4.9.2.3 Substrate specificity of GA-APase

The relative hydrolytic activity of GA-APase towards various substrates is shown in Table XVII. The enzyme was most active towards p-nitrophenyl phosphate followed by ATP, GTP, UTP and CTP. Glucose-6-phosphate, (Glc-6-p), glucose-1-phosphate (Glc-1-p), glyceraldehyde-3-phosphate (Gly-3-p), and 5´-mononucleotides were not hydrolyzed while ADP and GDP were hydrolyzed only very slightly, between 10 to 15%.

4.9.2.4 Effect of some metal cations and anions

The effect of some common metal cations and anions on GA-APase using pNPP as substrate was investigated. The results are summarized in Table XVIII. It was found that the enzyme had no metal ion requirement. In fact, in the presence of EDTA, there was about 23% enhancement in the enzyme activity and even Ca^{2+}, Mg^{2+} and Mn^{2+} inhibited the enzyme from 22 to 30 percent. K^{+} and Na^{+} had no effect on the enzyme. These results indicate that the GA-APase in not a Ca^{2+}-, Mg^{2+}-, Na^{2+}-, or K^{+}-ion dependent ATPase. Hg^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} were found to be potent inhibitors of the enzyme. The percentage inhibition of the enzyme activity was 100, 80, 62.5 and 81.6 respectively. Among the anions, F^{-}, PO_4^{3-} and $Mo_7O_{24}^{6-}$ were strong inhibitors of the GA-APase. Citrate and tartarate did not affect the enzyme activity.

4.9.2.5 Glycoprotein nature of GA-APase

The purified GA-APase was found to contain nearly 19 $\pm$ 1.5 percent carbohydrate. Treatment of the enzyme with periodate

TABLE XVII : Substrate specificity of GA-APase

The enzyme preparation (0.2 ml) was incubated with a final substrate concentration of 1.0 mM in a final volume of 1.5 ml 50 mM acetate buffer, pH 5.0. After incubation for 30 min at 30°C, the reaction was terminated with 0.5 ml of cold 20% trichloroacetic acid. Denatured proteins (if any) were removed by centrifugation and Pi released was determined in the supernatant by the method of Fiske-SubbaRow (82). The rate of hydrolysis for p-nitrophenyl phosphate was taken as 100%.

Substrate	Rate of hydrolysis µMPi/min/mg protein	Relative rate of hydrolysis[a] %
p-NPP	450.0	100.0
CTP	135.0	30.0
ATP	167.0	37.1
GTP	144.0	32.0
UTP	144.0	32.0
AMP		
GMP		
CMP		
UMP		
ADP	60.0	13.3
GDP	47.0	10.4
Glc-1-P		
Glc-6-P		
Gly-3-P		

a Relative rates were calculated relative to pNPP.

TABLE XVIII : Effect of some common metal ions and anions on the activity of Golgi apparatus-APase

Enzyme assays were performed without and with 10 mM metal ions and anions using standard reaction mixture.

Addition	Enzyme activity μMpNP/min/mg protein	% of Control
None	680.0	100.0
Na^{+}	685.0	100.8
K^{+}	687.0	101.0
Mg^{2+}	474.0	69.7
Mn^{2+}	490.0	72.1
Ca^{2+}	528.0	77.6
Zn^{2+}	136.0	20.0
Hg^{2+}	0.0	0.0
Cu^{2+}	255.0	37.5
Ni^{2+}	125.0	18.4
F^{-}	0.0	0.0
$Mo_7O_{24}^{6-}$	0.0	0.0
PO_4^{3-}	60.0	8.8
Citrate	683.0	100.4
Tartarate	687.0	101.0
EDTA	836.0	122.9

TABLE XIX : Effect of periodate oxidation on the activity of Golgi apparatus-APase from pea cotyledons

The purified GA-APase was incubated with and without $NaIO_4$ (10 mM) in 50 mM sodium acetate buffer, pH 5.0, for indicated periods in the dark at 4°C, and the APase activity was assayed after the addition of ethylene glycol and dialysis against the incubation buffer.

Incubation period (min)	Residual APase activity[a]			
	+ $NaIO_4$		-$NaIO_4$	
	µMpNP/min/mg protein, ±SD	Percent	µMpNP/min/mg protein, ±SD	Percent
0	673.0 ± 30	100	673.0 ± 26	100
15	600.9 ± 25	89.8	669.1 ± 24	99.4
30	417.9 ± 23	63.8	654.6 ± 24	97.2
60	267.2 ± 18	41.9	637.4 ± 22	94.7
120	68.6 ± 9	10.7	640.7 ± 22	95.2

a Results are average of three experiments and values are percent of controls ran concurrently.

(Table XIX) for 2 h resulted in 90 percent loss of activity, indicating the role of the carbohydrate for the activity of the GA-APase. The endo-H treatment of GA-APase (Fig 4.32) released about 50% of the total carbohydrate in 20 h. From these results, it appears that part of the carbohydrate is of high mannose type linked to the asparagine residue of protein. It is interesting to note that as the deglycosylation increases, the enzyme activity decreases (Fig 4.32). These results lend further support to the observation that the carbohydrate moiety is required for the activity of the GA-APase. An examination of the oligosaccharides released from the GA-APase by endo-H digestion on Bio-Gel P-4 column (Fig 4.33) shows the presence of heterogenous oligosaccharides with $GlcNAcMan_8$, the most dominant component, followed by $GlcNAcMan_9$, $GlcNAcMan_7$ and $GlcNAcMan_6$. Since only 50 percent of the total carbohydrate present in GA-APase was released by endo-H, at the moment, we are not certain about the nature of the remainder 50%.

4.9.2.6 Stability

The purified GA-APase was quite unstable and 50 percent activity was lost within 48 h at $0^{o}C$. In the presence of 25% glycerol, however, the enzyme was stable for about more than a month.

4.10 In vivo effect of monensin on the level of APase in various subcellular fractions

Monensin is a low molecular weight monovalent ionophore,

FIG 4.32 : Effect of deglycosylation by endo-H on the Golgi apparatus-APase from pea cotyledons

The purified enzyme was incubated with endo-H at a concentration of 50 mU/ml enzyme for the periods indicated and then analyzed for the APase activity. Control incubations were without endo-H. Experimental details were as described for Fig 4.23.

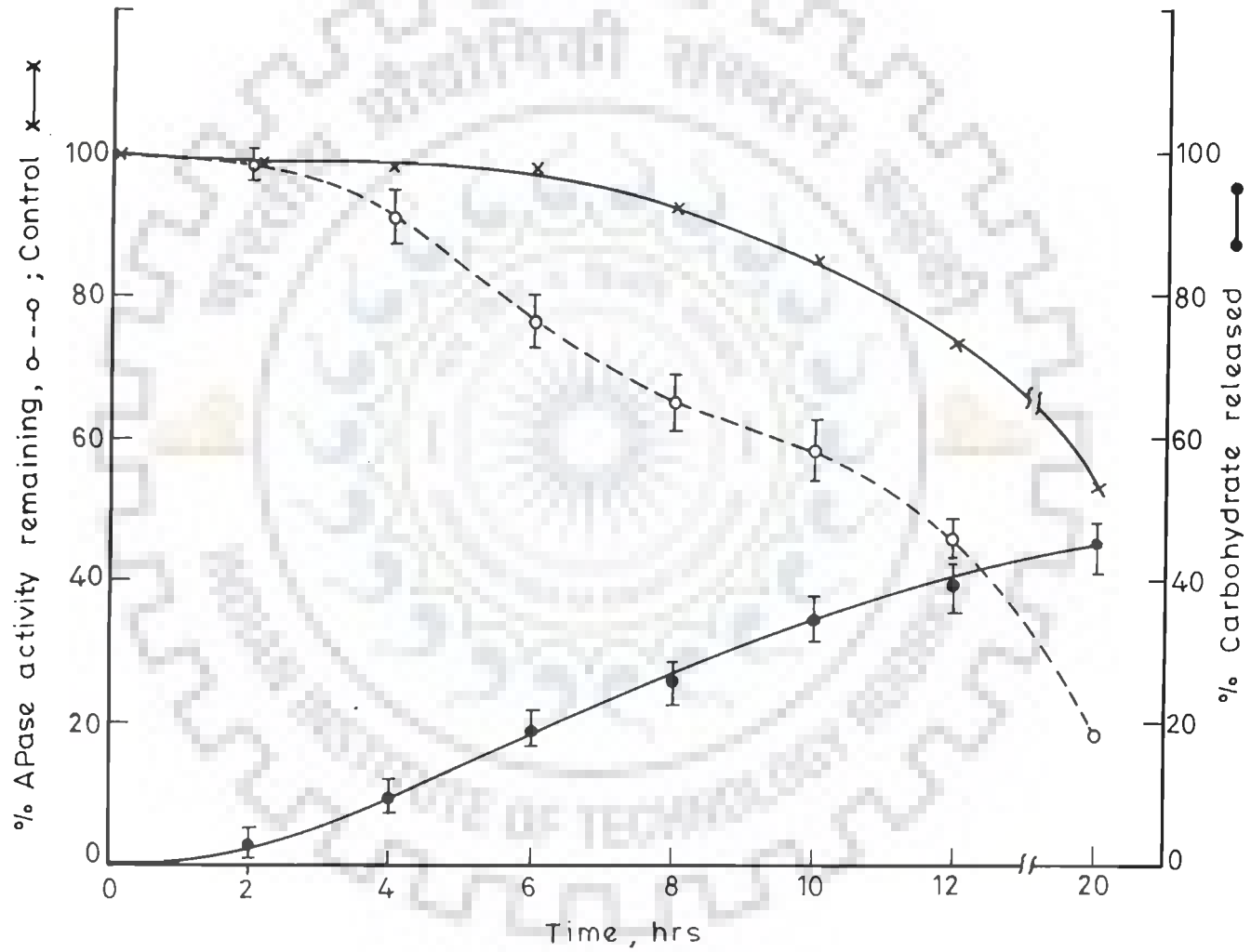
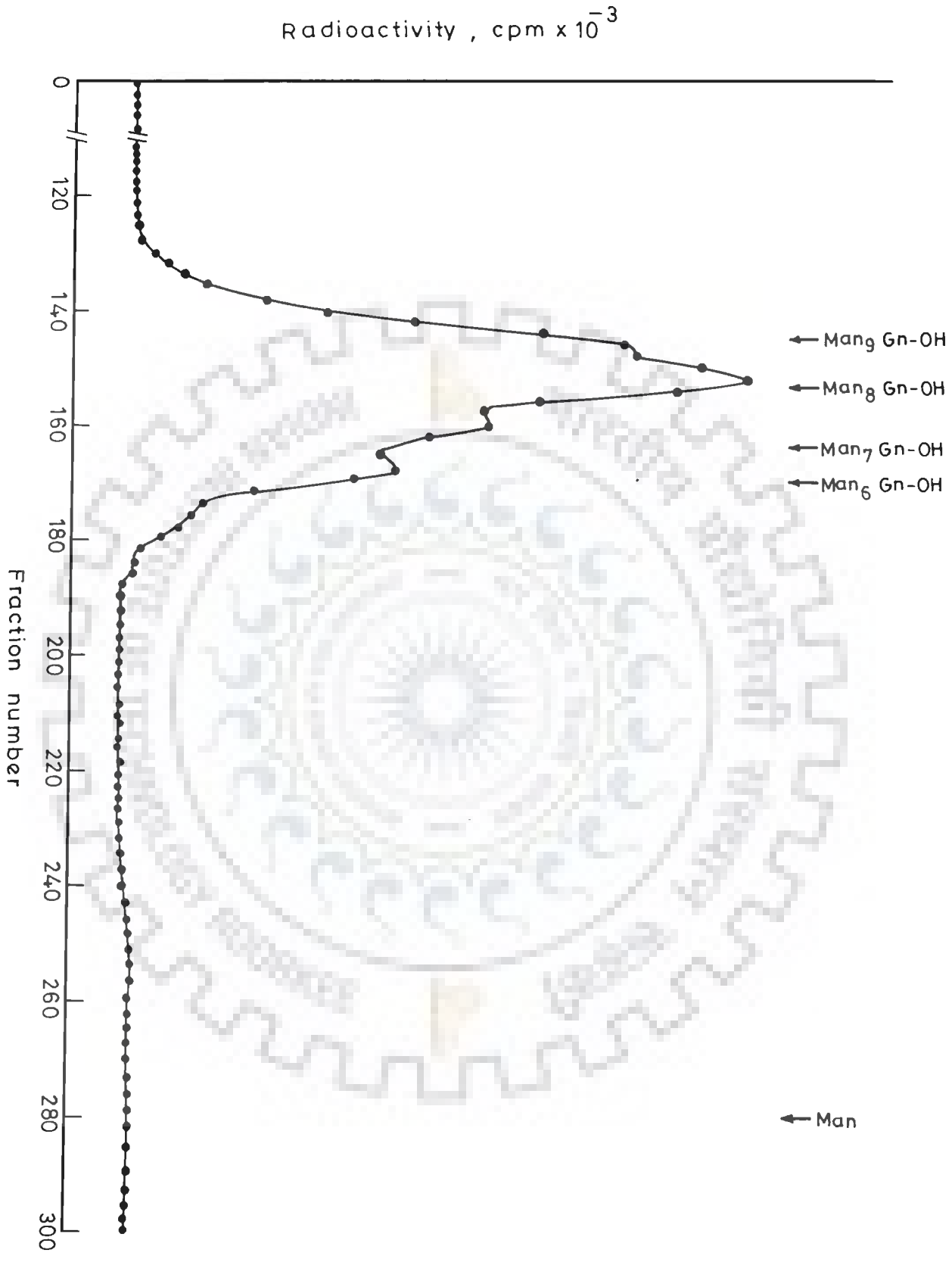

% APase activity remaining, o--o ; Control x—x
% Carbohydrate released
Time, hrs
0
20
40
60
80
100
0
2
4
6
8
10
12
20

FIG 4.33 : Bio-Gel P-4 chromatography of the oligosaccharides released by the endo-H treatment of the GA-APase

Experimental conditions were as those described in Fig 4.22.


Radioactivity , cpm x 10^{-3}
0
120
140
160
180
200
220
240
260
280
300
Fraction number
← Man_9 Gn-OH
← Man_8 Gn-OH
← Man_7 Gn-OH
← Man_6 Gn-OH
← Man

which interferes with the function of the Golgi apparatus of animal cells, has also been shown to interfere with the normal functioning of the Golgi apparatus in bean cells (54). In order to test the role of th Golgi apparatus in the transport of PM-APase, the effect of monensin on the intracellular distribution of acid phosphatase in pea cotyledons was examined. Pea seeds were first germinated without monensin for 4 days, the physiological state of the seed at which synthesis of microsomal APase occurs fairly rapidly, and then transferred to a medium containing 150 μM monensin. After 48h incubation, the cotyledons were separated from the seedlings and various subcellular fractions were prepared and APase activity was determined. The results (Table XX) showed that monensin inhibited the overall intracellular distribution of the enzyme with approximately 40 and 32% decrease in cytosolic and total crude microsomal (12,000 - 105,000 xg) APase activity. Among PM, GA and ER, the effect of monensin on PM-APase and GA-APase activities was comparable with 30% and 32% inhibition, respectively. However, the ER-APase activity was affected by about 19%. From these results, it appears that in pea cotyledons monensin is affecting the intracellular distribution of APase in a differential manner. It may be pointed out, however, that the results are not conclusive and more data are required. Monensin, further was found to have a profound retarding effect on the growth of the germinating seedings (Fig 4.34).

TABLE XX : In vivo effect of monensin on the APase activity in various membrane fractions

Pea seeds were germinated for 4 days under aseptic conditions as described in `Methods´. The germinating seedlings were then transferred to a medium containing 150 µM monensin and incubated for further 48 h. Controls were treated in same way, but without monensin in the medium. After the 48 h monensin treatment, 400 cotyledons (ca 80 g fw), from both treated and control, were collected at random and various subcellular fractions were prepared and APase activity was determined as described in `Methods´.

Fraction	APase activity, µM pNP/min/mg protein, [a] ± SD		Percentage of control
	Control (without monensin)	Monensin treated	
Cytosol	15.1 ± 5	9.0 ± 3	59.6
Crude microssomes (12,000-105,000xg pellet	16.0 ± 5	10.8 ± 3	67.5
Plasma membranes	40.5 ± 7	28.4 ± 5	70.1
Golgi apparatus	23.0 ± 4	15.6 ± 3	67.8
Endoplasmic reticulum	26.9 ± 4	21.7 ± 4	80.7

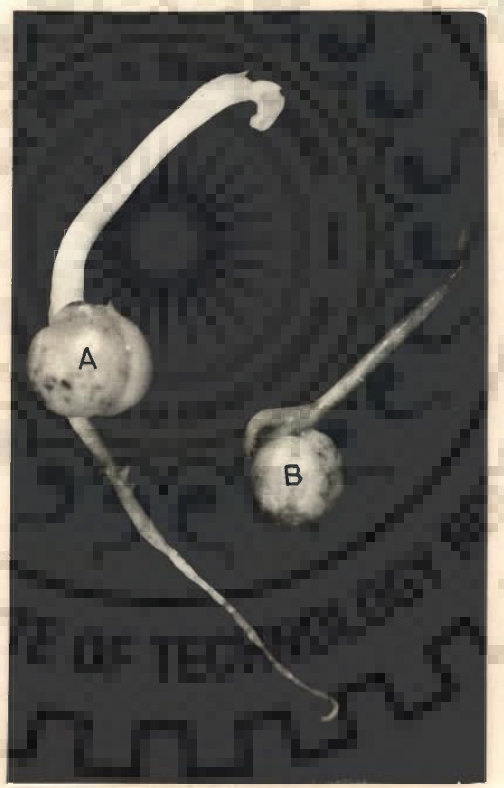
a Average of three separate experiments.

FIG 4.34 : **Effect of monensin on the growth of the germinating pea seedlings**

Pea seeds were germinated under aseptic conditions as described under `Methods´ for four days. The germinating seedlings were then transferred to a medium containing 150 uM monensim and incubated for further 48 h, controls were treated in the same way but without monensin in the medium

A control

B monensin-treated


A
B

5.0 DISCUSSION

The main purpose of the present study was to purify and characterize acid phosphatase (APase) from different endomembrane fractions of pea seeds with a view to use the enzyme as a model protein for studying the intracellular transport of proteins that are destined to become part of the plasma membrane in plant storage cells. As a first step, we prepared highly enriched PM, GA and ER fractions that were practically free from cross contaminations as judged by the absence of specific marker enzymes. For instance, the PM fraction did not show any significant activity of either glucan synthase I (GS-I) or inosine diphosphatase (IDPase), the marker enzymes for GA (99, 205), and of mannosyltransferase (MTase), marker enzyme for ER (153), indicating that the PM fraction was essentially free from GA and ER. Likewise, GA was free from PM and ER and ER was free from GA and PM. The marker enzyme used for the PM was glucan synthaseII (GS-II), a widely used PM marker enzyme (102, 205). Thus, it was considered reasonable to assume that the APase isolated from the purified PM, GA and ER used here was the true representative of the fraction from which the enzyme was isolated.

One of the major problems in the purification of APase was the removal of 5´-nucleotidase as the two enzymes have similar solubilizing characteristics (233) and also the fact that in general APase exhibit 5´-nucleotidase activity (122, 165, 257). Likewise, the 5´-nucleotidases show significant APase activity

(46, 233). However, this problem could be overcome as the developmental patterns of 5′-nucleotidase and APase in germinating pea cotyledons (Fig 4.3) were substantially different. For instance, whereas after 18 h imbibition of seeds, 5′-nucleotidase (measured as adenosine 5′-monophosphatase) was entirely lacking in the microsomal fraction, the level of APase in microsomes was fairly high. Thus, this differential developmental patterns of APase and 5′-nucleotidase was used for the isolation and purification of APase by taking 18h imbibed seeds to prepare the various endomembrane fractions which were almost completely devoid of 5′-nucleotidase activity. In addition, the level of other common acid phosphatases like glucose-6-phosphatase and glyceraldehyde-3-phosphatase was also very low compared to that of arylphosphatases (Table VII).

The intracellular distribution data (Table VI) showed the presence of APase in all the three endomembrane fractions (PM, GA and ER) each açcounting for about 35, 4.5 and 6.4 percent of the total microsomal APase activity. These values are, however, not absolute since a significant amount of membrane fractions, especially that of GA and ER, was discarded during purification. Nevertheless, the results are indicative of the relative abundance of APase in different fractions. Since we propose to use APase as a model protein for studying the intracellular transport of proteins in plant seeds, it is important to note that significant amount of APase activity was associated with PM, GA and ER, which are part of the secretory pathway. Further,

the membrane-bound APase especially the PM-APase, was fairly stable, thus allowing the purification of the enzyme.

The purification scheme described here is relatively simple involving selective solubilization of APase from the membranes by 1% CHAPS in the presence of 5 mM EDTA and a protein-to-detergent ratio of 2 : 3, pH precipitation and ion exchange chromatography on DEAE-Sephadex and CM-Sephadex and gel filtration in place of DEAE-Sephadex in the case of GA-APase. The enzyme purified by this procedure was homogeneous by SDS-PAGE although the purification of PM-APase and GA-APase were 95.8 and 54.6 folds with 1.1 and 0.73 percent yield, respectively. The actual purification fold and percent yield will be much greater, but firstly, because of the presence of substantial amount of other non-specific phosphatases in the crude microsomal fraction which was used as the basis of purification, and secondly, in the final stages of purification the enzyme becomes unstable resulting in lower specific activity. As per one estimate, as much as three fourths of the total phosphatase activity is accounted for by non-specific phosphatase activity (46). That the membrane-bound APase catalyzed the hydrolysis of more different phosphorylated substrates than the purified enzyme further supports the presence of several other non-specific APase in the crude microsomes.

The selective solubilization of APase by CHAPS was the first purification step. CHAPS was found to be an ideal detergent for releasing the membrane-bound APases into solution,

since it has a high critical micelle concentration (CMC) and, therefore, could be removed rapdily by dialysis without causing any loss in enzyme activity (108). Under optimum conditions of solubilization, the purification folds for the PM-APase and GA-APase were 7.9 and 5.3 respectively.

The results of ion exchange chromatography or gel filtration revealed the presence of multiple ionic species of APase in PM and GA of pea cotyledons. This is not unusual, however, as the plant seeds have been shown to contain APase isoenzymes (17, 81, 119, 192). In 18 h imbibed pea cotyledons, only one form of APase was found dominant. In the present study only the dominant species of APase has been purified and investigated further.

Properties of the membrane-bound and the purified PM-APase resemble closely in terms of pH optima, pH stability, kinetic parameters, sensitivitiy towards various metal ions and anions like Pi, $Mo_7O_{24}^{6-}$ and F^-. The only differences that were noticed between the membrane-bound and the purified APase were as following: The purified APase was not stimulated by citrate and tartarate ions, showed greater substrate specificity and much lower stability than the membrane-bound enzyme. As for the citrate and tartarate ion stimulation and the broader specificity of membrane-bound enzyme, the data support further that some other phosphatases or isoenzymes were present in the PM which were subsequently removed during the purification, since the effect of tartarate ions ranges from inhibitory (81) to stimulatory (52), to noneffective (51). Lower stability of the

purified APase also suggests that some stabilizing factors, like phospholipids, were lost during the purification in addition to the disruption of the natural environment in the membranes responsible for the greater stability of the membrane-bound enzyme (17, 233). Nevertheless, the purified enzyme represents a major PM-APase whose native structure, for all practical purposes appeared to have been maintained during the purification.

Acid phosphatases from different plant sources, possess different properties and a generalization is difficult to make. Nevertheless, by a broad definition, APases hydrolyse a variety of phosphomonoesters in acid pH (111), with non-specific substrate specificity. But the membrane-bound APase shows some specificity. Pea cotyledon plasma membrane APase does not hydrolyse phosphorylated sugars and mononucleotides, but is substantially active towards di-and trinucleotides. As suggested by Kamenan and Diopoh (122), the activity of the plasma membrane APase towards nucleotides may be necessary for the phosphorylation reaction and also to provide the energy necessary for the transport mechanism in which the enzyme may take part.

The effect of metal ions and anions on plant APases is so diverse that a generalization is also difficult to make. The pea cotyledon PM-APase, like many plant APases (81, 121, 122, 163, 242), is strongly inhihited by some common divalent cations (Zn^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}) monovalent anions (F^{-}) and polyvalent anions (PO_4^{3-}, $Mo_7O_{24}^{6}$). But unlike the tobacco protoplast APase (161), it is not activated by Ca^{2+} and ;Mg^{2+}. Nor does it

resemble some APase isoenzymes from rice endosperm which are stiumulated by Cu^{2+}, Mg^{2+} and Zn^{2+} (126). In fact, there is a significant inhibition by these cations of the pea cotyledon PM-APase. The effect of Zn^{2+} on PM-APase of pea cotyledons is different from the membrane-bound APases in bean hypocotyl (165), in being more potent in this case. From these data it seems that the response of the membrane-bound APases varies with the plant sources/tissues. Hence, if the tissue is the same, then a comparison of metal ion action on the enzyme from various endomembranes, like PM, GA and ER, would provide useful information about their structural and functional relationships.

The inhibition of APase by Pi is normally of competitive nature (81, 95, 122, 192, 207, 250) and so was the inhibition of the pea cotyledon PM-APase which is in agreement with earlier report (173, 255). However, the latter was different from the peanut cotyledon PM-APase (17), in which case the inhibition was of the noncompetitive mode. The Pi inhibition is of physiological importance and it may be involved in the regulation of energy metabolism.

The pea cotyledon PM-APase is a glycoprotein containing about 21% carbohydrate. Thus the enzyme confirms the general nature of plant APases with varying carbohydrate contents. In the latter regard, the pea enzyme with relatively low carbohydrate content compares well with APase from maize scutellum (218, 250), sweet potato (88), sycamore cell wall (62), yam tuber (122) and rye germ (81), but differs from the peanut

cotyledon PM-APase that contains about 50% carbohydrate (17), and also from several fungal APases (133, 162, 230, 275).

Endo-N-acetyl- β-D-glucosaminidase H (endo-H) treatment showed that at least three fourths of the carbohydrate moiety of the APase was linked to the peptide chain through N-glycosidic linkage involving N-acetyl-D-glucosamine and L-asparagine and is of high mannose type. N-glycosidic linkage have been shown to be present in fungal APases (230). An examination of the oligosaccharides released by endo-H action indicate, by no means prove, that oligosaccharides containing $Man_{9-6}GlcNAc_2$ were present. Since endo-H treatment would not release from proteins, the oligosaccharides containing $Mannose_{5-1}GlcNAc_2$, they may also be present. In any case, these results show a type of endo-H mapping of the pea cotyledon PM-APase which was useful for comparison with the enzyme from the other endomembrane (GA) fraction. Further precise work on characterization of endo-H-released oligosaccharides will be necessary. It may be pointed out here that at this moment, very little is known about the absolute structure of carbohydrates of plant glycoproteins in general (231) and membrane glycoproteins in particular (35).

The evidence provided by periodate oxidation and endo-H deglycosylation of the APase indicates that carbohydrates are necessary for the enzyme activity (XIV and XV). Since, APase activity was substantially reduced compared to the controls for APase, most of the oligosaccharides seem to be required for the APase activity. The carbohydrates in the plasma membrane-

associated glycoproteins are known to occur on the surface and probably act as a molecular shield blocking the access of proteases to the potential proteolytic cleavage sites or stabilize the conformation of the protein part in which the cleavage sites are exposed (208, 228). In conclusion, the pea cotyledon PM-APase is a glycoprotein with the major part of the carbohydrate moiety susceptible to endo-H indicating N-linked high mannose type and the enzyme requires the carbohydrate units for its activity. Whether glycosylation is also required for its transport from ER, site of synthesis, to the PM, is not known. We plan to study this aspect in cell cultures using tunicamycin, a specific inhibitor of N-glycosylation (251).

A considerable amount of APase activity was associated with the GA. Since the GA is involved in the processing and intracellular transport of macromolecules in both plant and animal cells and it receives the biosynthetic products from the ER which are destined to become part of the PM (79, 171, 189, 245), the GA-APase may be the precursor (or same) of the PM-APase. Using GA, free from PM as judged from the absence of marker enzyme of PM, APase was purified and its properties were compared with that of the PM-APase. The purification procedure followed was essentially the same as used for the PM-APase purification. Thus, it was assumed that the APases obtained separately from GA and PM were representatives of the source from which the enzyme was isolated.

As is evident from the properties of PM and GA-APases (Table XXI), the two enzymes resemble closely with respect to pH-

TABLE XXI : Comparison of properties of the purified plasma membrane and Golgi apparatus APases of pea cotyledons

Properties[a]	PM-APase	GA-APase
Molecular weight, kD		
(i) Gel filtration	(i) 69-70	65.5
(ii) SDS-PAGE	(ii) 68.0	61.1
Subunits present	single polypeptide	single polypeptide
Carbohydrate content, %	21.1	19.0
pH-optimum	5.2-5.6	5.2-5.6
Km (pNPP), M	3.1×10^{-4}	3.6×10^{-4}
Vmax (pNPP), $mM.min^{-1}.mg^{-1}$protein	2.0	0.87
Stability at $-20^{o}C$	Stable for 1 week	Stable for 3 days
Inhibition by F^{-}	non-competitive	non-competitive
Inhibition by NaH_2PO_4	Competitive	Competitive
Substrate specificity (relative hydrolysis rates,%)		
pNPP	100	100
ATP, UTP, CTP, GTP	30-40	35-40
ADP, GDP, UDP	30-40	10-15
AMP, GMP, UMP, CMP	0.0	0.0
Phosphorylated sugars	0.0	0.0
Metal ion requirements	None	None
Inhibition by metal ions, 10mM, %		
Hg^{2+}	95.1	100

TABLE XXI : (Contd.)

Properties[a]	PM-APase	GA-APase
Mn^{2+}	67.0	27.9
Cu^{2+}	97.0	62.5
Zn^{2+}	97.0	80.0
Ni^{2+}	40.0	81.7
Endo-H sensitivity		
(i) loss of activity in 8 h, percent	44.6	35.5
(ii) deglycosylation in 20 h, percent	72.0	45.0
Sensitivity to periodate activity loss in 2 h, %	83.0	89.3
In vitro inhibition by monensin, %	29.1	33.2

a Experiments for both PM and GA enzymes were carried out under identical conditions.

optimum, kinetic parameters, inhibition by F^-, PO_4^{3-} and $Mo_7O_{24}^{6-}$, substrate specificity, lack of metal ion requirement, inhibition by Hg^{2+}, Zn^{2+} and Cu^{2+}, sensitivity towards endo-H and periodate oxidation and the subunit structure. There are, however, some noticeable differences between the PM-and GA-APase. For instance, carbohydrate content and molecular weights, though small but are clearly significant. We believe that the 2% lower carbohydrate content of the GA-APase accounts for the lower molecular weight of GA-APase (65, 500 daltons) than the PM-APase (69,000 daltons). This is not usual since processing of carbohydrate moiety of glycoproteins also occurs in GA (60, 241, 245). Deglycosylation of PM-Pase and GA-APase by endo-H and the relationship between deglycosylation and enzyme activity of PM- and GA-enzymes are interesting and distinguishable characteristics that are probably related to the heterogeneity of the carbohydrate moiety in PM-and GA-APases introduced by post-translational processing in Golgi. The rate of deglycosylation of PM-APase is almost two times more than that of GA-APase. In addition, about 73% carbohydrate of purified PM-APase was endo-H sensitive compared to only 45% in GA-APase, indicating some major difference in the carbohydrate make up of the two enzymes, i.e. the PM-APase contains more of unmodified carbohydrate structures that meets the structural requirement of endo-H action than the GA-APase. Whether, this is a normal pattern and characteristic of PM- and GA-APases is not clear at the moment. The Bio-Gel P-4 patterns of the oligosaccharides released from the PM-APase and GA-APase by endo-H action show quantitative

difference in the relative abundance of oligosaccharides. Further studies with respect to the structure of oligosaccharides of PM-APase and GA-APase will be quite interesting. The present results, however, clearly show that N-linked oligosaccharides are required for the enzyme activity of both PM-and GA-APases. From the point of intracellular transport, the difference in oligosaccharide moiety is a useful parameter and makes APase a good candidate for studying the intracellular transport of proteins in seeds.

In conclusion, based on the comparison of purified PM-APase and GA-APase, the GA-APase seems to be the precursor enzyme with modified carbohydrate moiety, for the PM-APase. In view of the above it is suggested that the PM-APase may be used as a model glycoprotein for studying the intracellular transport of proteins from ER to PM in plant storage cells. In addition, it would also serve as a model enzyme to study the role of the carbohydrate moiety in the transport of APase from the ER to the GA and from the GA to the PM.

6. SUMMARY AND CONCLUSIONS

Various endomembrane fractions have been prepared from 18h imbibed pea cotyledons by combination of differential and sucrose density gradient centrifugations. Without Mg^{2+} and in the presence of 3 mM EDTA, organelles with average densities of 1.17 g/cm^3, 1.15 g/cm^3 and 1.10 g/cm^3 have been identified as membranes of PM, GA and ER, respectively, on the basis of their characteristic sedimentation properties and enrichment of the marker enzymes for PM (1,3- β-D-glucan synthase, GS-II), for GA (inosine diphosphatase, IDPase and 1, 4- β-D-glucan synthase, GS-I) and for ER (mannosyltransferase, MTase). PM, GA and ER thus obtained are enriched 12.8, 7 and 8 folds relative to the crude microsomes (12,000-105,000 xg pellet) and are free from cross contamination as judged by the presence/absence of marker enzymes specific for different endomembranes.

The microsomes account for at least 10 percent of the total APase activity in 18 h imbibed pea cotyledons and are devoid of 5´-nucleotidases and hexose phosphatases. Of the total microsomal APase activity about 35, 4.5 and 6.4 percent is associated with PM, GA and ER, respectively.

APase associated with PM, GA and ER shows remarkably similar properties with respect to pH-optimum, metal ion effect and response to inhibitors and activators. The noticeable difference is in their stabilities. The PM-APase exhibited greater stability than that of either GA or ER.

A major acid phosphatase (APase) has been purified to homogeneity from the PM of pea cotyledon by selective solubilization of the membrane-bound enzyme with 1 percent CHAPS at a protein-to-detergent ratio of 2:3 in the presence of 5mM EDTA, followed by ion exchange chromatography on DEAE-Sephadex, acid precipitation at pH 5.0 and CM-Sephadex chromatography. Both native and sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) of the enzyme revealed the presence of a single polypeptide band of around 69,000 daltons molecular weight. The pea cotyledon PM-APase is different from the peanut PM-APase in the sense that it does not show subunit structure (17). The enzyme has a slightly broad optimum pH between pH 5.2 and 5.6 and displays maximum stability between pH 4.8 and 6.4. The PM-APase shows no metal ion requirement and is powerfully inhibited by Hg^{2+}, Cu^{2+}, Zn^{2+} but only slightly inhibited by Mg^{2+}, Ni^{2+}, Mn^{2+} and Ca^{2+}. Orthophosphate and fluoride also inhibit the enzyme in a competitive and a noncompetitive manner, respectively. Under a set of defined conditions, the enzyme exhibits Km and Vmax values for p-nitrophenyl phosphate (pNPP) of 3.1×10^{-4}M and 2mM. min^{-1}. Mg^{-1} protein, respectively. Besides pNPP, the PM-APase also hydrolyses nucleoside di and nucleoside trisphosphates, but with only 30-40 percent efficiency. Nucleoside 5´-monophosphates, glucose-6-phosphate, glyceraldehyde-3-phosphate are not hydrolysed.

The PM-APase is a glycoprotein with 21.1 carbohydrate. On treatment with endo-N-acetyl- β -D-glucosaminidase H (endo-H), about 70 percent carbohydrate has been released from the enzyme,

indicating that the carbohydrate is linked to the peptide chain through N-glycosidic linkage between the amide nitrogen of asparagine and C_1 of N-acetylglucosamine and is of high mannose type. Both periodate treatment and deglycosylation of the enzyme by endo-H resulted in loss of enzyme activity. These results show that the carbohydrate moeity is required for the enzyme activity of PM-APase.

The Golgi apparatus-APase has been purified to homogeneity using an essentially similar purification scheme as has been used for the PM-APase. The only exception is the use of Sephadex G-100 gel filtration in place of DEAE-Sephadex. The properties of the pruified GA-APase resemble closely with those of the PM-APase except that the GA-APase is less stable, and the carbohydrate content (19 percent) and molelcular weight (65.5 kD) are slightly lower than those of the purifed PM-APase. The GA enzyme is also sensitive to endo-H treatment and periodate oxidation, although the degree of sensitivity is relatively smaller than the PM-APase. The results, however, indicate the presence of N-glycosidically linked oligosaccharides and its requirement for the enzyme activity of the GA-APase.

Based on the close resemblance between the PM-APase and GA-APase, it is suggested that the GA-APase may be a precursor of PM-APase in pea cotyledon cells and that it may be used as a model glycoprotein enzyme for studying the intracellular transport of proteins that are destined to become PM associated proteins in pea cotyledon cells.

7.0 REFERENCES

1. Adeli, K. and Altosaar, I. (1983) Role of endoplasmic reticulum in biosynthesis of oat globulin precursors. Plant Physiol. 73, 949 - 955.

2. Akazawa, T. and Miyata, S. (1983) Biosynthesis and secretion of α-amylase and other hydrolases in germinating cereal seeds. Essays in Biochemistry 18, 41 - 78.

3. Akiyama, T. and Suzuki, H. (1980) The release of acid phosphatase in wheat half-seed treated with gibberellic acid. Biochem. Physiol. Pflanz. 175, 436 - 446.

4. Akiyama, T., Uehimiya, H. and Suzuki, H. (1981) Gibberellic acid-induced increase in activity of a particular isoenzyme of acid phosphatase in wheat half-seeds. Plant Cell Physiol. 22, 1023 - 1028.

5. Akiyama, H. and Suzuki, H. (1981) Inhibitory effects of abscisic acid on gebberellic acid - induced acid phosphatase activity in wheat half-seeds. Plant Sci. Lett. 21, 137 - 143.

6. Ali., Mohd. S. and Akazawa, T. (1986) Association of H^+-translocating ATPase in Golgi membrane system from suspension-cultured cells of sycamore (Acer pseudoplatanus L.). Plant Physiol. 81, 222 - 227.

7. Ali. Mohd. S., Nishimura, M., Mitsui, T., Akazawa, T. and Kojima, K. (1985) Isolation and characterization of Golgi membranes from suspension-cultured cells of sycamore (Acer pseudoplatanus L.). Plant Cell Physiol. 26, 1119-1133.

8. Anderson, R.L. and Ray, P.M. (1978) Labeling of the plasma membrane of pea cells by a surface-localized glucan synthetase. Plant Physiol. 61, 723 - 730.

9. Ashford, A.E. and Jacobson, J.V. (1974) Cytochemical localization of phosphatase in barley aleurone cells : the pathway of gibberellic acid - induced enzyme release. Planta 120, 81 - 105.

10. Babszinski, P. and Tanner, W. (1978) A membrane associated isoenzyme of invertase in yeast precursor of the external glycoprotein. Biochem. Biophys. Acta 548, 426 - 436.

11. Badet, J. and Jeanloz, R.W. (1988) Isolation and partial purification of lipid-linked oligosaccharides from calf pancreas. Carbohydrate Research 178, 49 - 65.

12. Bailey, C.T., Cobb, A. and Boulter, D. (1970) A cotyledon slice system for the autoradiographic study of the synthesis and intracellular transport of the seed storage proteins of Vicia faba. Planta 95, 103 - 118.

13. Bailey, K.M., Phillips, I.D.J. and Pitt, D. (1976) Effects of gibberellic acid on the activation, synthesis and release of acid phosphatase in barley seeds. J. Expt. Bot. 27, 324 - 336.

14. Baker, J.E. and Takeo, T. (1973) Acid phosphatase in plant tissues. 1-Changes in activity and multiple forms in tea leaves and tomato fruit during maturation and senescence. Plant Cell Physiol. 14, 459 - 471.

15. Barrett-Lennard, E.G., Robson, A.D. and Greenway, H. (1982) Effect of phosphorus deficiency and water deficit on phosphatase activities from wheat leaves. J. Expt. Bot. 33, 682 - 693.

16. Barrett--Lennard, E.G. and Greenway, H. (1982) Partial separation and characterization of soluble phosphatases from leaves of wheat grown under phosphorus deficiency and water deficit. J. Expt. Bot. 33, 694 - 704.

17. Basbaa, O., Das, J. and Sharma, C.B. (1987) Solubilization, purification and characterization of an acid phosphatase from the plasma membrane of peanut cotyledons. Ind. J. Biochem. Biophys. 24, 24 - 28.

18. Bennett, A.B., O´Neil, S. and Spanswick, R.M. (1984) H^+-ATPase activity from storage tissue of Beta vulgaris. 1. Identification and characterization of anion-sensitive H^+ATPase. Plant Physiol. 74, 538 - 544.

19. Bergman, L.W. and Kuehl, W.M. (1978) Temporal relationship of translocation and glycosylation of immunoglobulin heavy and light chain. Biochemistry 17, 5174 - 5180.

20. Besford, R.T. (1979) Quantitative aspects of leaf acid phosphatase and the phosphorus status of tomato plants. Ann. Bot. 44, 153 - 161.

21. Bhargava, R. and Sachar, R.C. (1983) Chloramphenicol stimulates acid phosphatase activity in germinating cotton (Gyossypium hirsutum) embryos. Biochem. J. 212, 73 - 77.

22. Biswas, T.K., Panigrahi, S.S., Mondal, N.K. and Dube, D.K. (1979) Acid phosphatase activity in cotyledons of germinating seeds of Vigna sinensis (Linn) savi. Curr. Sci. 48, 301 - 303.

23. Blobel,G. and Dobberstein, B. (1975) Transfer of proteins across membranes I. J. Cells Biol. 67, 835 - 851.

24. Blobel, G. and Dobberstein, B. (1975) Transfer of proteins across membranes 2. J. Cell Biol. 67, 852 - 862.

25. Blumwald, E., Fortin, M.G., Rea, P.A., Verma, D.P.S. and Poole, R.J. (1985) Presence of host-plasma membrane type H^+-ATPase in the membrane envelope enclosing the bacterioids in soybean root nodules. Plant Physiol. 78, 665 - 672.

26. Boer, P. and Steyn-Parve´, E.P. (1966) Isolation and purification of acid phosphatase from baker´s yeast (Saccharomyces cerevisiae). Biochem. Biophys. Acta 128, 400 - 402.

27. Boer, P., Van Rijn, J.M., Reinking, A. and Steyn-Parve´, E.P. (1975) Biosynthesis of acid phosphatase of baker´s yeast. Characterization of a protoplast-bound fraction containing precursors of the exoenzyme. Biochem. Biophys. Acta 377, 331 342.

28. Bollini, R. and Chrispeels, M.J. (1979) The rough endoplasmic reticulum is the site of reserve-protein synthesis in developing Phaseolus vulgaris cotyledons. Planta 146, 487 - 501.

29. Bollini, R., Ceriotti, A., Daminati, M. and Vitale, A. (1985) Glycosylation is not needed for the intracellular transport of phytohemagglutinin in developing Phaseolus vulgaris cotyledons and for the maintenance of its biological activities. Physiol. Plantarum 65, 15 - 22.

30. Bollini, R., Van der Wilden, W. and Chrispeels, M.J. (1982) A precursor of the reserve protein, phaseolin, is transiently associated with the endoplasmic reticulum of developing Phaseolus vulgaris cotyledons. Plant Physiol. 55, 82 - 92.

31. Bonner, J. (1965) Methods of cell fractionation. In `Plant Biochemistry´ (Bonner, J. and Varner, J.E. eds.) pp. 7-12, Academic Press, New York.

32. Boulau, E.R., Sabatin, D.D., Pereyra, B.N. and Kreibich, G. (1978) Spacial orientation of glycoproteins in membranes of rat liver rough microsomes. J. Cell Biol. 78, 894 - 909.

33. Bowden, L. and Lord, J.M. (1978) Purification and comparative properties of microsomal and glyoxysomal malate synthase from castor bean endosperm. Plant Physiol. 61, 259 - 265.

34. Bowles, D.J., Lehle, L. and Kauss, H. (1977) Glycosylation of sterols and polyprenolphosphate in the Golgi apparatus of Phaseolus aureus. Planta 134, 177 - 181.

35. Bowles, D.J. (1982) Membrane glycoproteins. In `Encyclopedia of Plant Physiology´. New Series. (Loewus, F.A. and Tanner, W. eds) Vol. 13A, pp. 584 - 660. Springer-Verlay, Berlin, Heidelberg, New York.

36. Briskin, D.P. and Poole, R.J. (1983) Characterization of a K^+-stimulated adenosine triphosphatase associated with the plasma membrane of red beet. Plant Physiol. 71, 350-355.

37. Briskin, D.P. and Thornley, W.R. (1985) Plasma membrane ATPase of sugar beet. Phytochemistry 24, 2797 - 2802.

38. Brown, J.W.S., Ersland, D.R. and Hall, T.C. (1982) Molecular aspects of storage protein synthesis during seed development. In `The Physiology and Biochemistry of seed development, dormancy and germination´. (A.A. Khan, ed.) pp. 3 - 42, Elsevier Biochemical Press, Amsterdam.

39. Buchanan, B.B., Nancy, A.C. and Wolosiuk, R.A. (1979) Activation of plant acid phosphatases by oxidized glutathione and dehydroascorbate. Plant Sci. Lett. 14, 245 - 251.

40. Buckhout, T.J., Heyder-Caspers, L. and Sievers, A. (1982) Fractionation and characterization of cellular membranes from root tips of garden cress (Lepidium sativum L.) Planta 156, 108 - 116.

41. Buckhout, T.J. (1983) ATP-dependent Ca^{2+} transport in endoplasmic reticulum isolated from roots of Lepidium sativum L. Planta 159, 84 - 90.

42. Buckhout, T.J. (1984) Characterization of Ca^{2+} transport in purified endoplasmic reticulum membrane vesicles from Lepidium sativum L. roots. Plant Physiol. 76, 962 - 967.

43. Bush, D.R. and Sze, H. (1986) Calcium transport in tonoplast and endoplasmic reticulum vesicles isolated from cultrued carrot cells. Plant Physiol. 80, 549 - 555.

44. Buvat, R. and Robert, G. (1979) Vacuole formation in the actively growing root meristems of barley (Hordeum sativum). Am. J. Bot. 66, 1219 - 1237.

45. Caro, L.G. and Palade, G.E. (1964) Protein synthesis, storage and discharge in the pancreatic exocrine cells. J. Cell Biol. 20, 473 - 495.

46. Carter, S.G. and Tipton, C.L. (1986) Purification and characterization of 5´-nucleotidase from Zea mays microsomes. Phytochemistry 25, 33 - 37.

47. Chanson, A., McNaughton, E. and Taiz., L. (1984) Evidence of a KCl-stimulated Mg^{2+}-ATPase on the Golgi of corn coleoptiles. Plant Physiol. 76, 498 - 507.

48. Chanson, A. and Taiz. L. (1985) Evidence for an ATP-dependent proton pump on the Golgi of corn coleoptiles. Plant Physiol. 78, 232 - 240.

49. Chanson, A., Fichmann, J., Spear, D. and Taiz, L. (1985) Pyrophosphate-driven proton transport by microsomal membranes of corn coleoptiles. Plant Physiol. 79, 159-164.

50. Chanson, A. and Pilet, P-E. (1987) Localization in sucrose gradients of the pyrophosphate-dependent proton transport of maize root membranes. Plant Physiol. 84, 1431 - 1436.

51. Chen, S-C., Ogura, N., Nakagawa, H. and Takehana, H. (1975) Purification and some properties of two acid phosphatases from tomato fruit. Agric. Biol. Chem. 39, 2069 - 2070.

52. Ching, T-M., Lin, T.P. and Metzger, R.T. (1987) Purification and properties of acid phosphatase from plump and shriveled seeds of triticate. Plant Physiol. 84, 789 - 795.

53. Chrispeels, M.J. (1980) The endoplasmic reticulum. In `The biochemistry of plants´ (Stumpf, P.K. and Conn. E.E. eds.) Vol. 1, pp. 389 - 412. Academic Press, New York.

54. Chrispeels, M.J. (1983) The Golgi apparatus mediates the transport of phytohemagglutinin to the protein bodies in bean cotyledons. Planta 158, 140 - 151.

55. Chrispeels, M.J., Higgins, T.J.V., Craig, S. and Spencer, D. (1982) The role of the endoplasmic reticulum in the synthesis of reserve proteins and the kinetics of their transport to protein bodies in developing pea cotyledons. J. Cell Biol. 93, 5 - 14.

56. Chrispeels, M.J. (1985) UDP-GlcNAc : glycoprotein GlcNAc-transferase is located in the Golgi apparatus of developing bean cotyledons. Plant Physiol. 78, 835 - 838.

57. Cohen, R.E. and Ballou, C.E. (1981) Mannoprotein structure. In `Enyclopedia of Plant Physiology´. New Series. (Tanner, W. and Loewus, F.A. eds.) Vol. 13B, pp.441 - 458. Springer-Verlag, Berlin, Heidelberg and New York.

58. Condor, M.J. and Lord, J.M. (1983) Heterogeneous distribution of glycosyltransferases in the endoplasmic reticulum of castor bean endosperm. Plant Physiol. 72, 547 - 552.

59. Cornshaw, J. and Bentwood, B.J. (1977) Cytochemical localization of β-glycerophosphatase in the xylem of Nicotiana tobacum and Pisum sativum. Biol. Cell 28, 65 - 73.

60. Craig, S. and Goodchild, D.J. (1984) Golgi-mediated vicilin accumulation in pea cotyledons is redirected by monensin and nigricin. Protoplasma 122, 91 - 97.

61. Crasnier, M. and Giordani, R. (1985) Elution of acid phosphatase from sycamore cell walls. Plant Sci. 40, 35 - 41.

62. Crasnier, M., Noat, G. and Ricard, J. (1980) Purification and molecular properties of acid phosphatase from sycamore cell walls. Plant Cell Environ. 3, 217 - 224.

63. Churchill, K.A. and Sze. H. (1983) Anion-sensitive, H^+-pumping ATPase in membrane vesicles from oat roots. Plant Physiol. 71, 610 - 617.

64. Churchill, K.A., Holaway, B. and Sze. H. (1983) Separation of two types of electrogenic H^+-pumping ATPase from oat roots. Plant Physiol. 73, 921 - 928.

65. Dauwalder, M., Whale, W.G. and Kephart, J.E. (1969) Phosphatases and differentiation of the Golgi apparatus. J. Cell Sci. 4, 455 - 497.

66. De Leo, P. and Sacher, J.A. (1970) Senescence. Association of synthesis of acid phosphatase with banana ripening. Plant Physiol. 46, 208 - 211.

67. De Michelis, M.I. and Spanswick, R.M. (1986) H^+-pumping driven by the vanadate-sensitive ATPase in membrane vesicles from corn roots. Plant Physiol. 81, 542 - 547.

68. Dieckert, J.W. and Dieckert, M.C. (1976) The chemistry and cell biology of vacuolar proteins in seeds. J. Food Sci. 41, 475 - 482.

69. Dominy, P.J. and Heath, R.L. (1985) Inhibition of K^+-stimulated ATPase of the plasmalemma of pinto bean leaves by ozone. Plant Physiol. 77, 43 - 45.

70. Dracup, M.N.H., Barrett-Lennard, E.G., Greenway, H. and Robson, A.D. (1984) Effect of phosphorus deficiency on phosphatase activity of cell walls from roots of subterranean clover. J. Expt. Bot. 35, 466 - 480.

71. Dubios, M., Gilles, K.A., Hamilton, J.K., Robers, P.A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350 - 356.

72. DuPont, F.M. and Leonard, R.T. (1980) Solubilization and partial purification of adenosine triphosphatase from a corn root plasma membrane fraction. Plant Physiol. 65, 931 - 938.

73. DuPont, F.M., Burke, L.L. and Spanswick, R.M. (1981) Characterization of a partially purified adenosine triphosphatase from corn root plasma membrane fraction. Plant Physiol. 67, 59 - 63.

74. DuPont, F.M. and Hurkman, W.J. (1985) Separation of the Mg^{2+}-ATPases from the Ca^{2+}-phosphatase activity of microsome membranes prepared from barely roots. Plant Physiol. 77, 857 - 862.

75. DuPont, F.M. (1987) Variable effects of nitrate on ATP-dependent proton transport by barley root membranes. Plant Physiol. 84, 526 - 534.

76. Elbein, A.D. (1982) The role of lipid linked saccharides in the biosynthesis of complex carbohydrates. In `Encyclopedia of Plant Physiology´. New Series. (Tanner, W. and Loewus, F.A. eds.) Vol. 13A, pp. 166 - 193. Springer-Verlag, Berlin, Heidelberg, New York.

77. Eylar, E.H. (1965) On the biological role of glycoproteins J. Theor. Biol. 10, 89 - 113.

78. Falk, R.H. and Stocking, C.R. (1976) Plant membranes. In `Encyclopedia of plant physiology´. New Series. (Stocking, C.R. and Heber, U. eds.) Vol. 3, pp. 3 - 50. Springer-Verlag, Berlin, Heidelberg, New York.

79. Faye, L., Ghorbel, A. and Mouatassim, B. (1984) Glycosylation and intracellular transport : the example of radish β -fructosidase. Physiol. Veg. 22, 351 - 364.

80. Faye, L., Greenwood, J.S., Herman, E.M., Sturm, A. and Chrispeels, M.J. (1988) Transport and Posttranslational processing of the vacuolar enzyme α -mannosidase in jack-been cotyledons. Planta 174, 271 - 283.

81. Ferens, M. and Morawiecka, B. (1985) Rye germ acid phosphatase : properties of the enzyme and its activation by lectins. Phytochemistry 24, 2839 - 2842.

82. Fiske, C.H. and SubbaRow, Y. (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375 - 400.

83. Flinn, A.M. and Smith, D.L. (1967) The localization of enzymes in cotyledons of Pisum arvense L. during germination. Planta 75, 10 - 22.

84. Folch, J., Lees, M. and Stanley, G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 266, 497 - 509.

85. Forti, G., Tognoli, C. and Parish, B. (1962) Purification from pea leaves of a phosphatase that attacks nucleotides. Biochim. Biophys. Acta 62, 251 - 260.

86. Fujimoto, S., Nakagawa, T. and Ohara, A. (1977) Isolation of violet-colored acid phosphatase from soybean. Agric. Biol. Chem. 41, 599-600.

87. Fujimoto, S., Naagawa, T. and Ohara, A. (1977) Further studies on the properties of violet-coloured acid phosphatase from soybean. Chem. Pharm. Bull. 25, 3283 - 3288.

88. Fujimoto, S., Ohara, A. and Uehara, K. (1980) Carbohydrate and metal analysis of violet colored acid phosphatase of sweet potato. Agric. Biol. Chem. 44, 1659 - 1660.

89. Gabard, K.A. and Jones, R.L. (1986) Localization of phytase and acid phosphatase isoenzymes in aleurone layers of barley. Physiol. Plantarum 67, 182 - 192.

90. Gabathuler, R. and Cleland, R.E. (1985) Auxin regulation of a proton translocating ATPase in pea root plasma membrane vesicles. Plant Physiol. 79, 1080 - 1085.

91. Gallagher, S.R. and Leonard, R.T. (1982) Effect of vanadate, molybdate and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots. Plant Physiol. 70, 1335 - 1340.

92. Gardiner, M. and Chrispeels, M.J. (1975) Involvement of the Golgi apparatus in the synthesis and secretion of hydroxyproline-rich cell wall glycoproteins. Plant Physiol. 55, 536 - 541.

93. Garoff, H. and Schwarz, R.T. (1978) Glycosylation is not necessary for membrane insertion and cleavage of Semliki Forest virus membrane proteins. Nature (London) 274, 487 - 490.

94. Gibson, R., Schlessinger, S. and Kornfled, S. (1979) The nonglycosylated glycoprotein of vesicular stomatitisvirus is temperature sensitive and undergoes intracellular aggregation at elevated temperatures. J. Biol. Chem. 254, 3600 - 3607.

95. Giordani, R., Nari, J., Noat, G. and Sauve, P. (1986) Purification and molecular properties of an acid phosphatase from Asclepias curassavia latex. Plant Sci. 43, 207 - 212.

96. Goldstein, A.H., Baertlein, D.A. and McDaniel, R.G. (1988) Phosphate starvation inducible metabolism in Lycopersicon essulentum. I. Excretion of acid phosphatase by tomato plants and suspension-cultured cells. Plant Physiol. 87, 711 - 715.

97. Goldstein, A.H., Danon, A. Baertlein, D.A. and McDaniel, R.G. (1988) Phosphate starvation inducible metabolism in Lycopersicon esculentum. II Characterization of the phosphate starvation inducible-exerted acid phosphatase. Plant Physiol. 87, 716 - 720.

98. Gonzalez, E. (1982) Aggregated forms of malate and citrate synthase are localized in the endoplasmic reticulum of germinating castor bean. Plant Physiol. 69, 83 - 87.

99. Green,J.R. (1983) The Golgi apparatus. In `Isolation of membranes and organelles from plant cells´ (Hall, J.L. and Moore, A.L. eds.) pp. 135 - 152. Academic Press, New York.

100. Griffing, L.R. and Quatrano, R.S. (1984) Isolectric focusing of plant cell membranes. Proc. Natl. Acad. Sci. U.S.A. 81, 4804 - 4808.

101. Gupta, A.K., Bhatia, I.S. (1980) Purification and characterization of acid phosphatase from Aspergillus niger tieghem. Ind. J. Microbiol. 19, 71 - 74.

102. Hall, J.L. (1983) Plasma membranes. In `Isolation of membranes and organelles from plant cells´(Hall, J.L. and Moore, A.L. eds.) pp. 55 - 81. Academic Press, New York.

103. Haris, N. (1979) Endoplasmic reticulum in developing seeds of Vicia faba, Planta 146, 63 - 69.

104. Hasegawa, Y., Lynn, K.R. and Brockbank, W.J. (1976) Isolation and partial characterization of cytoplasmic and wall-bound acid phosphatases of wheat roots. Can. J. Bot. 54, 1163 - 1169.

105. Herman, E.M. and Shannon, L.M. (1985) Accumulation and subcellular localization of α -galactosidase-hemagglutinin in developing soybean cotyledons. Plant Physiol. 77, 886 - 890.

106. Hickman, S. and Neufeld, E.F. (1972) A hypothesis for cell disease detective by hydrolases that not enter lysosomes. Biochem. Biophys. Res. Commun. 49, 992 - 999.

107. Hickman, S., Shapiro, L.J. and Neufeld, E.F. (1974) A recognition marker required for uptake of a lysosomal enzyme. Biochem. Biophys. Res. Commun. 57, 55 - 61.

108. Hjelmeland, L. and Chrambach, A. (1984) Solubilization of functional membrane proteins. Method Enzymol. 104, 305 - 328.

109. Hodges, T.K., Leonard, R.T., Bracker, C.E. and Keenan, T.W. (1972) Purification of anion stimulated adenosine triphosphatase from plant roots : association of plasma membranes. Proc. Natl. Acad. Sci. U.S.A. 69, 3307 - 3311.

110. Hodges, T.K. and Mills, D. (1986) Isolation of the plasma membrane. Methods Enzymol. 118, 41 - 45.

111. Hollander, V.P.(1971) Acid Phosphatase, In `The enzymes' (Boyer P.D. eds.) Vol. 4, pp. 449 - 498. Academic Press, New York.

112. Holley, R. (1984) Gibberellic acid controls the secretion of acid phosphatase in aleurone layers and isolated aleurone protoplasts of Avena fatua. J. Expt. Bot. 35, 822 - 828.

113. Holley, R. (1984) Gibberellic acid controls specific acid-phosphatase isozymein aleurone cells and protoplasts of Avena fatua L. Planta 161, 355 - 360.

114. Huang, A.H.C. (1975) Comparative studies of glyoxysomes from various fatty seedlings. Plant Physiol. 55, 870 - 874.

115. Hubbard, S.C. and Ivatt, R.J. (1981) Synthesis and processing of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 50, 555 - 583.

116. Igaue, I., Watabee, H., Takahashi, K., Takeheshi, M. and Morota, A. (1976) Violet-colored acid phosphatase isozymes associated withcell wall preparations from rice plant cultured cells. Agric. Biol. Chem. 40, 823 - 825.

117. Imbrie, C.W. and Murphy, T.M. (1984) Solubilization and partial purification of ATPase from a rose cell plasma membrane fraction. Plant Physiol. 74, 611 - 616.

118. Ishikawa, M. and Yoshida, S. (1985) Seasonal changes in plasma membranes and mitochondria isolated from Jerusalem artichoke tubers. Possible relationship to cold hardiness. Plant Cell Physiol. 26, 1331 - 1344.

119. Johnson, C.B., Holloway, B.R., Smith, H. and Grierson, D. (1973) Isoenzymes of acid phosphatase in germinating peas. Planta 115, 1 - 10.

120. Johnson, K.D. and Chrispeels, M.J. (1987) Substrate specificity of N-acetylglucosaminyl-, fucosyl- and xylosyl transferases that modify glycoprotein in the Golgi apparatus of bean cotyledons. Plant Physiol. 84, 1301 - 1308.

121. Kamenan, A., and Diopoh, J. (1982) Purification and physiochemical properties of an acid phosphatase from *Dioscorea cayenensis* cytoplasm. Plant Sci. Lett. 24, 173 - 182.

122. Kamenan, A. and Diopoh, J. (1983) Properties of two membrane-bound acid phosphatases compared with those of a cytoplasmic acid phosphatase from Dioscorea cayenensis rotundata. Plant sci. Lett. 32, 305 - 312.

123. Kaplan, A., Achard, D.T. and Sly, W.S. (1977) Phosphohexosyl components of a lysosome enzyme that are recognized by pinocytosis receptors on human fibroblasts Proc. Natl. Acad. Sci. U.S.A. 76, 2026 - 2030.

124. Karr, A.L. (1972) Isolation of an enzyme system which will catalyze the glycosylation of extensin. Plant Physiol. 50, 275 - 282.

125. Kasamo, K. (1986) Purification and properties of the plasma membrane H^+-translocating adenosine triphosphatase of Phaseolus mungo L. roots. Plant Physiol. 80, 818 - 824.

126. Kawanobe, H. (1977) Acid phosphatase in the endosperm of rice seed. Diagaku Kyoikugakubu Kenkyn, Shizen Kagaku, 27, 72 - 77 (Japan).

127. Kiely, M.L., Mcknight, G.S. and Schimke, R.T. (1976) Studies on the attachment of carbohydrate to ovalbumin nascent chains in hen oviduct. J. Biol. Chem. 251, 5490 - 5495.

128. Kleppinger-Sparce, K.F. and Moore, T.S., Jr. (1985) Biosynthesis of cytidine 5′-diphosphatediacylglycerol in endoplasmic reticulum and mitochondria of castor bean endosperm. Plant Physiol., 77, 12 - 15.

129. Koehler, D.E., Leonard, R.T., Van der Woude, W.J., Linkins, A.E. and Lewis, L.N. (1976) Association of latent cellulase activity with plasma membranes from kidney bean abscision zones. Plant Physiol. 58, 324-330.

130. Konopska, L. and Sakowski, R. (1978) Enzyme activities in aleurone grains of pea seeds during early stages of germination. Biochem. Physiol. Pflanz. 173, 536 - 540.

131. Kornfled, R. and Kornfled, S. (1976) Comparative aspects of glycoprotein structure. Annu. Rev. Biochem. 45, 217 - 237.

132. Korner, L.E., Kjellbom, P., Larsson, C. and Moller, I.M. (1985) Surface properties of right side-out plasma membrane vesicles isolated from barley roots and leaves. Plant Physiol. 79, 72 - 79.

133. Krasnopevtseva, N.G., Padkina, M.V., Atanasov, B.K. and Smirnov, M.N. (1979) Genetic-biochemical study of acid phosphatase from Saccharomyces cerevisiae. III. Isolation and characterization of acid phosphatase. Vestn Leningr. Univ. Biol. 3, 97 - 103 (U.S.S.R.).

134. Kubowicz, B.D., Van der Hofe, L.N. and Hanson, J.B. (1982) ATP-dependent calcium transport in plasmalemma preparations from soybean hypocotyls. Effect of hormone treatment. Plant Physiol. 69, 187 - 191.

135. Kuo, S.C. and Lapman, C. (1974) Tunicamycin : an inhibitor of yeast glycoprotein synthesis. Biochem. Biophys. Res. Commun. 58, 287 - 295.

136. Lall, S., Berry, M., Saluja, D. and Sachar, R.C. (1988) Differential rates of ∝-amylase and acid phosphatase induction by GA_3 in embryoless half-seeds and isolated aleurones of wheat. Plant Sci. 55, 185 - 190.

137. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature (London) 22, 680 - 685.

138. Lamport, D.T.C. and Catt, J.W. (1981) Glycoprotein and enzymes of the cell wall. In `Encyclopedia of plant physiology´. New Series. (Tanner, W. and Loewus, F.A. eds.) Vol. 13 B, pp. 131 - 165. Springer-Verlag, Berlin.

139. Larsson, C. (1985) Plasma membrane. In `Modern methods of plant analysis. Cell components´ (Linskens, H.E. and Jackson, J.F. eds.) Vol. 1, pp. 85 - 104. Springer-Verlag, Berlin.

140. Leavitt, R., Schlesinger, S. and Kornfled, S. (1977) Impaired intracellular migration and altered solubility of non-glycosylated glycoproteins of USV and Sindbis virus. J. Biol. Chem. 252, 9018 - 9023.

141. Lehle, L., Fartaczek, F., Tanner, W. and Kauss, H. (1976) Formation of polyprenol-linked mono-and oligosaccharides in Phaseolus aureus. Arch. Biochem. Biophys. 175, 419 - 426.

142. Lehle, L. (1981) Biosynthesis of mannoproteins in fungi. In `Encyclopedia of Plant Physiology´. New Series. (Tanner, W. and Loewus, F.A. eds.) Vol. 13 B, pp. 459 - 483. Springer-Verlag. Berlin, New York.

143. Leigh, R.A. and Walker, R.R. (1980) ATPase and acid phosphatase activities associated with vacuoles isolated from storage roots of red beet (Beta vulgaris L.). Planta 150, 222 - 229.

144. Leonard, T.L. and Hotchkiss, C.W. (1976) Cation - stimulated adenosine triphosphatase activity and cation transport in corn roots. Plant Physiol. 58, 331 - 335.

145. Leonard, R.T. and Hodges, T.K. (1980) The plasma membrane. In `The biochemistry of plants´ (Stumpf, P.K. and Conn, E.E. eds.) Vol. I, pp. 163 - 182. Academic Press, New York.

146. Lercher, M., Wojciechowski, Z.A. (1976) Localization of plant UDP-glucose : sterol glucosyltransferase in the Golgi membranes. Plant Sci. Lett. 7, 337 - 340.

147. Linnemans, W.A.M., Boer, P. and Elbers, P.F. (1977) Localization of acid phosphatase in Saccharomyces cerevisiae : a clue to cell wall formation. J. Bacteriol. 131, 638 - 644.

148. Loomis, W.D. (1974) Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol. 31A, 528 - 544.

149. Lorenc-Kubis, I. and Morawiecka, B.C. (1978) Phosphatase activity of Poa pratensis seeds. III. Effect of fluoride, citrate, urea and other substances on the activity of acid phosphatases Ia_2, Ia_3. Acta Soc. Bot. Pol. 47, 83 - 89.

150. Lorenc-Kubis, I. (1986) Some properties and immunological relationship of acid phosphatases from Gramineae. Plant Sci. 44, 1 - 5.

151. Lord, J.M., Kagawa, T., Moore, T.S. and Beevers, H. (1973) Endoplasmic reticulum as a site of lethicin synthesis in castor bean endosperm. J. Cell Biol. 57, 659 - 667.

152. Lord, J.M. and Bowden, L. (1978) Evidence that glyoxysomal malate synthase is segregated by the endoplasmic reticulum. Plant Physiol. 61, 266 - 270.

153. Lord, J.M., (1983) Endoplasmic reticulumn and ribosomes. In `Isolation of membranes and organelles from plant cells´ (Hall, J.A. and Moore, A.L. eds.) pp. 119 - 134. Academic Press, New York.

154. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265 - 275.

155. Lugon, M. Goldberg, R. and Guen, J. (1974) Evidence for the intervention of cell wall phosphatases in AMP utilization by Acer pseudoplatanus cells. Plant Sci. Lett. 3, 165 - 171.

156. Marty, F., Branton, D. and Leigh, R.A. (1980) Plant vacuoles. In `The biochemistry of plants´ (Stumpf, P.K. and Conn, E.E. eds.) Vol. I, pp. 625 - 656. Academic Press, New York.

157. Matile, P., Cortat, M., Wiemken, A. and Frey-Wyssling, A. (1971) Isolation of glucanase containing particles from budding Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 68, 636 - 640.

158. Matsumoto, H. and Yamaya, T. (1984) Repression of the K^+ uptake and cation-stimulated ATPase activity associated with the plasma membrane-enriched fraction of cucumber roots due to Ca^{2+} starvation. Plant Cell Physiol. 25, 1501 - 1511.

159. Melroy, D. and Jones, R.L. (1986) The effect of monensin on the intracellular transport and secretion of α -amylase isoenzymes in barley aleurone. Planta. 167, 252 - 259.

160. McCarty, D.R., Keegstra, K. and Selman, B.R. (1984) Characterization and localization of the ATPase associated with pea chloroplast envelope membranes. Plant Physiol. 76, 584 - 588.

161. Mettler, I.J. and Leonard, R.T. (1979) Isolation and Partial characterization of vacuoles from tobacco protoplasts. Plant Physiol. 64, 1114 - 1120.

162. Mildner, P., Barbaric, S., Kozulic, B. and Ries, B. (1980) Acid phosphatase, a glycoprotein of the yeast cell wall. Proc. FEBS Meet. (Enzyme Regul. Mech. Action) 60, 83-92.

163. Mittal, R., Das, J., and Sharma, C.B. (1988) Purification and characterization of 5´-nucleotidase from Golgi apparatus of peanut cotyledons. Plant Sci. 55, 95 - 101.

164. Miyata, S. and Akazawa, T. (1982) Enzymic mechanism of starch breakdown in germinating rice seeds. 12. Biosynthesis of α - amylase in relation to protein glycosylation. Plant Physiol. 70, 147 - 153.

165. Mizuta, S. and Suda, S. (1980) A comparative study of the multiple acid phosphatases in cellular fractions of bean hypocotyl. Ann. Bot. (London) 45, 369 - 382.

166. Mlodianowski, F. (1972) The occurrence of acid phosphatase in the thykaloids of developing plastids in lupin cotyledons. Z. Pflanzenphysiol. 66, 362 - 365.

167. Mollenhauer, H.H. and Morre, D.J. (1980) The Golgi apparatus. In `The Biochemistry of Plants´ (Stumpf, P.K. and Conn. E.E. eds.) Vol. I, pp. 437 - 488. Academic Press, New York.

168. Moor, H. (1967) Endoplasmic reticulum as the initiator of bud formation in yeast (S. cerevisiae). Arch. Microbiol. 57, 135 - 146.

169. Monique, C. (1979) Ultrastructural localization of acid phosphatase activity and phosphates in radicles of Allium cepa in ungerminated seeds and the beginning of germination. Effect of short treatment at low temperature (- 196°C). Ann. Sci. Nat. Bot. Biol. Veg. 1, 21 - 38, (French).

170. Moore, D.J. (1971) Isolation of Golgi apparatus. Methods Enzymol. 22, 130 - 148.

171. Moore, D.J. (1975) Membrane biogenesis. Ann. Rev. Plant Physiol. 26, 548 - 567.

172. Morre, D.J., Franke, W.W., Deumling, B., Nyquist, S.E. and Ovtracht, L. (1971) Golgi apparatus function in membrane flow. Biomembranes 2, 95 - 104.

173. Murray, D.R. and Collier, M.D. (1977) Acid phosphatase activities in developing seeds of Pisum sativum L. Aust. J. Plant Physiol. 4, 843 - 848.

174. Murray, D.R. (1980) Functional significance of acid phosphatase distribution during embryo development in Pisum sativum L. Ann . Bot. (London) 45, 273 - 281.

175. Nagahashi, J. and Beevers, L. (1978) Subcellular distribution of glycosyl transferases involved in glycoprotein biosynthesis in the cotyledons of Pisum sativum L. Plant Physiol. 61, 451 - 459.

176. Nagahashi, J. and Hiraike, K. (1982) Effect of centrifugal force and centrifugation time on the sedimentation of plant organelles. Plant Physiol. 69, 546 - 548.

177. Nagahashi, J. and Kane, A.P. (1982) Triton-stimulated nucleoside diphosphatase activity : subcellular localization in corn root homogenates. Protoplasma 112, 167 - 173.

178. Neufeld, E.F. (1979) Processing and recognition of lysosomal enzymes. In `Glycoconjugates´ Proc. 5th Int. Symp. Thieme, Stuttgart, pp. 320 - 321.

179. Neville, D.M. and Chang, T.M. (1978) Receptor-mediated protein transport into cells. Curr. Top. Membr. Transp. 10, 65 - 150.

180. Newton, R.P., Moyse, C.J., Coles, M.P. and Collin, P.J.D. (1979) Low-specificaty phosphatase isoenzymes in Phaseolus vulgaris. Biochem. Soc. Trans. 7, 1269 - 1271.

181. Ninomiya, Y., Ueki, K. and Sato, S. (1977) Chromatographic separation of extracellular acid phosphatase of tobacco cells cultured under Pi-supplied and omitted conditions. Plant Cell Physiol. 18, 413 - 420.

182. Novick, P. and Schekman, R. (1979) Secretion and cell-surface growth are blocked by temperature-sensitive mutant of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 76, 1858 - 1862.

183. O´Connell, A.M. and Groove, T.S. (1985). Acid phosphatase activity in karri (Eucalyptus diversicolor F. Muel.) in relation to soil phosphate and nitrogen supply. J. Expt. Bot. 36, 1359 - 1372.

184. Odds, F.C. and Hierholzer, J.C. (1973) Purification and properties of glycoprotein acid phosphatase from Candida albicans. J. Bacteriol. 114, 257 - 266.

185. Olden, L., Pratt, R.M. an Yamada, K.M. (1978) Role of carbohydrates in protein secretion and turn-over. Effect of tunicamycin on the major cell surface glycoprotein of chick embroyo fibroblast. Cell 13, 461 - 473.

186. O´Neil, S.D., Bennett, A.B. and Spanswick, R.M. (1983) Characterization of NO_3-sensitive H^+-ATPase from corn roots. Plant Physiol. 72, 837 - 846.

187. O´Neil, S.D. and Spanswick, R.M. (1984) Effect of vanadate on the plasma membrane ATPase of red beet and corn. Plant Physiol. 75, 586 - 591.

188. Oparka, J.K. and Johnson, R.P.C. (1982) Acid phosphatase activity in the intercellular spaces in roots of Nymphoides peltata (S.G. Gmel.) O. Kuntze. Plant Cell Environ. 5, 237 - 240.

189. Palade, G.E. (1975) Intracellular aspects of the process of protein synthesis. Sience 189, 347 - 358.

190. Pan, S-m (1987) Characterization of multiple acid phosphatase in salt-stressed spinach leaves. Aust. J. Plant Physiol. 14, 117 - 124.

191. Parodi, A.J. and Loloir, L.F. (1979) The role of lipid intermediate in the glycosylation of proteins in the eukaryotic cell. Biochem. Biophys. Acta 559, 1 - 37.

192. Park, H-s. C. and Van Etten, R.L. (1986) Purification and characterization of homogeneous sunflower seed acid phosphatase. Phytochemistry 25, 351 - 357.

193. Paul, E.M. and Williamson, V.M. (1987) Purification and properties of acid phosphatase-I from a nematode resistant tomato cultivar. Plant Physiol. 84, 399 - 403.

194. Perlin, D.S. and Spanswick, R.M. (1981) Characterization of ATPase activity associated with corn leaf plasma membranes. Plant Physiol. 68, 521 - 526.

195. Pierce, W.S. and Hendrix, D.L. (1979) Utilization of enzyme markers to determine the localization of plasma membrane from Pisum stivum epicotyls on sucrose gradient. Planta 146, 161 - 169.

196. Poole, R.J., Briskin, D.P., Kratky, Z. and Johnstone, R.M. (1984) Density gradient localization of plasma membrane and tonoplast from storage tissue of growing and dormant red beet. Characterization of proton-transport and ATPase in tonoplast vesicles. Plant Physiol. 74, 549 - 556.

197. Powell, J.T. and Brew, K. (1974) Glycosyl transferases in Golgi-membranes of onion stem. Biochem. J. 142, 203-209.

198. Price, C.A. (1983) General principles of cell fractionation. In `Isolation of membranes and organelles from Plant Cells' (Hall, J.L. and Moore, A.L. eds.) pp. 1 - 24. Academic Press, New York.

199. Pyliotis, N.A., Ashford, A.E., Whitecross, M.I. and Jacobson, J.V. (1979) Localization of gibberellic acid - induced acid phosphatase activity in the endoplasmic reticulumn ofbarley aleurone cells with the electron microscope. Planta 147, 134 - 140.

200. Quail, P.H. (1979) Plant cell fractionation. Ann. Rev. Plant Physiol. 30, 425 - 484.

201. Rainina, E.I., Subatov, A.S., Bukhvalov, I.B. and Luzikov, V.N. (1979) Cytochemical and biochemical determination of the activity of acid phosphatase. Tsitologiya 21, 1403 - 1410 (Russian).

202. Ramirez, J.M., Gallego, G.G. and Serrano, R. (1984) Electron transfer constituents in plasma membrane fractions of Avena sativa and Saccharomyces cerevisiae. Plant Sci. Lett. 34, 103 - 110.

203. Randall, S.K. and Ruesink, A.W. (1983) Orientation and integrity of plasma membrane vesicles obtained from carrot protoplasts. Plant Physiol. 73, 385 - 391.

204. Rasi-Caldogno, F., Pugliarello, M.C. and De Michelis, M.I. (1985) Electrogenic transport of proteins driven by the plasma membrane ATPase in membrane vesicles from radish. Biochemical characterization. Plant Physiol. 77, 200 - 205.

205. Ray, P.M. (1977) Auxin-binding sites of maize coleoptiles are localized on membranes of the endoplasmic reticulum. Plant Physiol. 59, 594 - 599.

206. Ray, P.M., Shininger, T.L. and Ray, M.M. (1969) Isolation of β-glucan synthetase particles from plant cells and identification with Golgi membranes. Proc. Natl. Acad. Sci. U.S.A. 64, 605 - 612.

207. Reid, M.S. and Bieleski, R.L. (1970) Changes in phosphatase activity in phosphorus-deficient Spirodela. Planta 94, 274 - 281.

208. Reinwald, E. (1985) Role of carbohydrates within variant surface glycoprotein of Trypanosoma congolense. Protection against proteolytic attack. Eur. J. Biochem. 151, 385 - 391.

209. Ribas, A.I. (1977) Relation between the acid phosphatase content and the germinating capacity of different types of seeds. Circ. Farm. 35, 341 - 355 (Spain).

210. Riorden, J.R. and Foster, G.G. (1978) Glycoprotein membrane enzymes. Curr. Top. Membr. Transp. 11, 146 - 231.

211. Robinson, D.G. and Glas, R. (1983) The glucan synthases from suspension-cultured sugar cane cells, J. Expt. Bot. 34, 668 - 675.

212. Robinson, M.L. and Carman, G.M. (1982) Solubilization of microsomal-associated phosphatidylinositol synthase from germinating soybeans. Plant Physiol. 69, 146 - 149.

213. Rodriguez, L., Ruiz, T., Villanueva, J.R. and Sentandreu, R. (1978) Yeast invertase : subcellular distribution and possible relationship between the isoenzymes. Curr. Microbiol. 1, 41 - 44.

214. Rodriguez, L., Ruiz, T., Elroza, M.V., Villanueva, J.R. and Sentandreu, R. (1980) Metabolic relationship between invertase and acid phosphatase isoenzymes in Saccharomyces cerevisiae. Biochem. Biophys. Acta 629, 445 - 454.

215. Rothman, J.E., Katz, F.N. and Lodish, H.F. (1978) Glycosylation of membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion a transmembrane protein. Cell 15, 1447 - 1454.

216. Rothman, J.E. and Fene, R.E. (1980) Coated vescicles transport newly synthesized membrane glycoprotein from endoplasmic reticulum to plasma membranes in two successive stages. Proc. Natl. Acad. Sci. U.S.A. 77, 780 - 784.

217. Rosenberg, I. and Holly, A. (1979) Nucleolytic enzymes of rape seedling. II. Non-specific acid phosphomonoester hydrolase. Collect. Czech Chem. Commun. 44, 3327 - 3340.

218. Rossi, A., Palma, M.S., Leone, F.A. and Brigliador, M.A. (1981) Properties of acid phosphatase from scutella of germinating maize seeds. Phytochemistry 20, 1823 - 1826.

219. Sanchez-Pina, M.A., Rodringues-Garcia, M.I. and Risueno, M.C. (1980) Localization of acid phosphatase activity in plant cell nucleoli. Cell Biol. Int. Rep. 4, 93 - 104.

220. Sandelius, A.S., Penel, C., Auderset, G., Brightman, A., Milland, M. and Morre, D.J. (1986) Isolation of highly purified fractions of plasma membrane and tonoplast from the same homogenate of soybean hypocotyls by free-flow electrophoresis. Plant Physiol. 81, 117 - 185.

221. Satoh, S. and Fujii, T. (1984) Presence of organelle-associated serine enzymes in spinach leaves. Plant Cell Physiol. 25, 63 - 67.

222. Satoh, S. and Fujii, T. (1985) Presence of serine enzymes in endoplasmic reticulum of spinach callus. Plant Cell Physiol. 26, 1037 - 1044.

223. Satoh, S. and Fujii, T. (1985) A membrane-bound protease in microsomes of spinach callus. Plant Physiol. 78, 267 - 271.

224. Schlesinger, S., Gottlieb, C., Feil, P., Gelb, N. and Kornfled, S. (1976) Growth of enveloped RNA viruses in a line of Chinese hamster ovary cells with deficient N-acetylglucosaminyl transferase activity, J. Virol. 17, 239 - 246.

225. Schonholzer, F., Schweingruber, A.M., Trachsel, H. and Schweingruber, M.E. (1985) Intracellular maturation and secretion of acid phosphatase of Saccharomyces cerevisiae. Eur. J. Biochem. 147, 273 - 279.

226. Schumaker, K.S. and Sze, H. (1985) A Ca^{2+}/H^{+} antiport system driven by the proton electrochemical gradient of a tonoplast ATPase from oat roots. Plant Physiol. 79, 1111 - 1117.

227. Schwaiger, H. and Tanner, W. (1979) Effect of gibberellic acid and tunicamycin on glycosyltransferase activities and an α-amylase secretion in barley. Eur. J. Biochem. 102, 375 - 381.

228. Schwarz, R.T. and Datema, R. (1982) Inhibition of lipid dependent glycosylation. In `The glycoconjugates´ (Horwitz, M.I. eds.) Vol. III, pp. 47 - 79. Academic Press, New York.

229. Schwarz, R.T., Rohrschneider, J.M. and Schmidt, M.F.G. (1976) Suppression of a glycoprotein formation of Semliki Forest, influenza and avian sarcome virus by tunicamycin. J. Virol. 19, 782 - 791.

230. Schweingruber, A.M., Schoenholzer, F., Keller, L., Schwaninger, R., Trachsel, H. and Schweingburger, M.E. (1986) Glycosylation and secretion of acid phosphatase in Schizosaccharomyces pombe. Eur. J. Biochem. 158, 133-140.

231. Selvendran, R.R. and O´Neill, M.A. (1982) Plant glycoproteins. In `Encyclopedia of Plant Physiology´. New Series. (Loewus, F.A. and Tanner, W. eds.) Vol. 13A, pp. 515-583. Springer-Verlag, Berlin, Heidelberg, New York.

232. Sentandreu, R., Larriba, G. and Elroza, M.V. (1981) Secretory processes-general considerations and secretion in fugi. In `Encyclopedia of Plant Physiology´. New Series. (Tanner, W. and Loewus, F.A. eds.) Vol. 13B, pp. 487 - 512. Springer-Verlag, Berlin.

233. Sharma, C.B., Mittal, R. and Tanner, W. (1986) Purification and properties of a glycoprotein adenosine - monophosphatase from the plasma membrane fraction of Arachis hypogea cotyledons. Biochem. Biophys. Acta 884, 567 - 577.

234. Shimada, Y., Shinmyo, A. and Enatsu, T. (1977) Purification and properties of one component of acid phosphatase produced by Aspergillus niger. Biochem. Biophys. Acta 480, 417 - 427.

235. Shinshi, H. and Kato, K. (1979) Purification and properties of acid phosphatase from cultured tobacco cells. Phytochemistry 18, 243 - 245.

236. Shore, G. and Maclachlan, G. (1975) The site of cellulose synthesis. Hormone treatment alters the intracellular location of alkali-insoluble β -1,4-glucan (cellulose) synthetase activities. J. Cell Biol. 64, 557 - 571.

237. Spiro, M.J., Spiro, R.J. and Bhoyro, V.D. (1979) Glycosylation of proteins by oligosaccharide-lipids. J. Biol. Chem. 254, 7668 - 7674.

238. Stinissen, H.M., Peumans, W.J. ande Chrispeels, M.J. (1985) Posttranslational processing of proteins in vacuoles and protein bodies is inhibited by monesin. Plant Physiol. 77, 495 - 498.

239. Struck, D.K., Sinta, P.B., Lane, M.M. and Lennarz, W.J. (1978) Effect of tunicamycin on the secretion of serum proteins by primary cultures of rat and chick hepatocytes. Studies on transferrin very low density lipoprotein and serum albumins. J. Biol. Chem. 253, 5332 - 5337.

240. Sturm, A. and Kindl, H. (1983) Fucosyl transferase and fucose incorporation in vivo as markers for subfractionating cucumber microsomes. FEBS Lett. 160, 165 - 168.

241. Sturm, A., Voelker, T.A., Herman, E.M. and Chrispeels, M.J. (1988) Correct glycosylation, Golgi processing and targetting to protein bodies of the vacuolar protein phytohemagglutinin in transgenic tobacco. Planta 175, 170 - 183.

242. Suzuki, T. and Sato, S. (1973) Properties of acid phosphatase in cell wall of tobacco cells cultured in vitro. Plant cell Physiol. 14, 585 - 596.

243. Szabo - Nagy, A., Olah, Z. and Erdei, L. (1987) Phosphatase induction in roots of winter wheat during adaptation to phosphorus deficiency. Physiol. Plantarum 70, 544 - 552.

244. Tabas, I. and Kornfled, S. (1978) The synthesis of complex type oligosaccharides. J. Biol. Chem. 253, 7779 - 7786.

245. Tabas, I. and Kornfeld, S. (1979) Purification and characterization of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. J. Biol. Chem. 254, 11655 - 11665.

246. Tabas, I., Schlesinger, S. and Kornfeld, S. (1978) Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and IgG Heavy Chain. J. Biol. Chem. 253, 716 - 722.

247. Taiz, L., Murray, M. and Robinson, D.G. (1983) Identification of secretory vesicles in homogenates of pea stem segments. Planta 158, 534 - 539.

248. Tamura, T., Ninamikawa, T. and Koshiba, T. (1982) Multiple forms of acid phosphatase in cotyledons of Vigna mungo seedlings. J. Expt. Bot. 33, 1332 - 1339.

249. Tan, K.K. (1978) Assays of proteins by Lowry method in samples containing 2-mercaptoethanol. Anal. Biochem. 86, 327 - 331.

250. Teno, A.M., Palma, M.S. and Rossi, A. (1987) Acid Phosphatase from maize scutellum : properties as a function of seed germination. Phytochemistry 26, 55 - 58.

251. Tkacz, J.S. and Lapman, J.O. (1975) Tunicamycin inhibition of poliisoprenol N-acetylglucosaminyl pyrophosphate formation in calf-liver microsomes. Biochem. Biophys. Res. Commun. 69, 248 - 257.

252. Toth, R. and Kujit, J. (1977) Cytochemical localization of acid phosphatase in endophyte cells of the semiparasite angiosperm Camandra umbellata (Santalaceae). Can. J. Bot. 55, 470 - 475.

253. Trimble, R.B. and Maley, F. (1977) Sub-unit structure of external invertase from Saccharomyces cerevisiae. J. Biol. Chem. 252, 4409 - 4412.

254. Turco, S.J. and Robbins, P.W. (1979) The initial stages of processing of protein bound oligosaccharides in vitro. J. Biol. Chem. 254, 4560 - 4567.

255. Turner, D.H. and Turner, J.F. (1960) The hydrolysis of glucose monophosphates by a phosphatase preparation from pea seeds. Biochem. J. 74, 486 - 491.

256. Uehara, K., Fujimoto, S. and Taniguchi, T. (1974) Studies on violet-colored acid phosphatase of sweet potato. I. Purification and some physical properties. J. Biochem. 75, 627 - 638.

257. Uehara, K., Fujimoto, S., Taniguchi, T. and Nakai, K. (1974) Studies on violet-colored acid phosphatase of sweet potato. II. Enzymatic properties and amino acid composition. J. Biochem. 75, 639 - 649.

258. Ueki, K. and Sato, S. (1971) Effect of inorganic phosphate on the extracellular acid phosphatase activity of tobacco cells cultured in vivo. Physiol. Plant 24, 506 - 511.

259. Uemura, M. and Yoshida, S. (1983) Isolation and identification of plasma membrane from light-grown winter rye seedlings (Secale cereale L. cv. puma). Plant Physiol. 73, 586 - 597.

260. Uemura, M. and Yoshida, S. (1985) Comparison of plasma membranes from winter rye (Secale cereale cv. puma) : Polypeptide composition, ATPase activity and specific naphthylphthalamic acid binding capacity. Plant Cell Physiol. 26, 35 - 46.

261. Van der Wilden, W., Gilkes, N.R. and Chrispeels, M.J. (1980) The endoplasmic reticulum of mung bean cotyledons. Role in the accumulation of hydrolases in protein bodies during seedling growth. Plant Physiol. 66, 390 - 394.

262. Van der Wilden, W. and Chrispeels, M.J. (1983) Characterization of the isoenzymes of α-mannosidase located in the cell wall, protein bodies and endoplasmic reticulum of Phaseolus vulgaris cotyledons. Plant Physiol. 71, 82 - 87.

263. Van Rijn, H.J.M., Boer, P. and Steyn-Parve, E.P. (1972) Biosynthesis of acid phosphatase of baker´s yeast. Factors influencing its production by protoplasts and characterization of the secreted enzyme. Biochim. Biophys. Acta 268, 431 - 441.

264. Van Rijn, H.J.M., Linnemans, W.A.M. and Boer, P. (1975) Localization of acid phosphatase in protoplasts from Saccharomyces cerevisiae. J. Bacteriol. 123, 1144 - 1149.

265. Von Figura, K. and Klein, V. (1979) Isolation and characterization of phosphorylated oligosaccharides from β-N-acetylglucosaminidase that are recognized by cell surface receptor. Eur. J. Biochem. 94, 347 - 354.

266. Yamaya, T., Tanigawa, M. and Matsumoto, H. (1982) Specific increase in phosphatase isozymes in cucumber roots caused by calcium deficiency. Plant cell Physiol. 23, 385 - 395.

267. Yamagata, H., Tanaka, K. and Kasai, Z. (1979) Isoenzymes of acid phosphatase in aleurone particles of rice and their interconversion. Agric. Biol. Chem. 43, 2059-2066.

268. Yamagata, H., Tanaka, K. and Kasai, Z. (1980) Purification and characterization of acid phosphatase in aleurone particles of rice grains. Plant Cell Physiol. 21, 1449 - 1460.

269. Yamagata, H., Sugimoto, T., Tanaka, K. and Kasai, Z. (1982) Biosynthesis of storage proteins in developing rice seeds. Plant Physiol. 70, 1094 - 1100.

270. Yang, C.M., Danko, S.J. and Markwell, J.P. (1987) Thylakoid acid phosphatase and protein phosphatase activities in wheat (Triticum aestivum L.) Plant Sci. 48, 17 - 22.

271. Yoshida, S., Uemura, M., Niki, T., Sakai, A. and Gusta, L.V. (1983) Partition of membrane particles in aqueous two-polymer phase system and its practical use for purification of plasma membranes from plants. Plant Physiol. 72, 105 - 114.

272. Yoshida, S. (1984) Chemical and biophysical changes in the plasma membrane during cold acclimation of mulberry bark cells (Morus bombycis Koidz. cv. Goroji). Plant Physiol. 76, 257 - 265.

273. Yoshida, S., Kawata, T., Uemura, M. and Niki, T. (1986) Properties of plasma membrane isolated from chilling-sensitive etiolated seedlings of Vigna radiata L. Plant Physiol. 80, 152 - 160.

274. Wagner, G.J. and Hrazdina, G. (1984) Endoplasmic reticulum as a site of phenylpropanoid and flavonoid metabolism in Hippeastrum. Plant Physiol. 74, 901 - 906.

275. Watorek, W., Morawiecka, B. and Rozen, K. (1977) Acid phosphatase in the yeast Rhodotorula ruba. Purification and properties of the enzyme. Acta Biochem. Pol. 24, 153 - 162.

276. Whitman, C.E. and Travis, R.L. (1985) Phospholipid composition of a plasma membrane-enriched fraction from developing soybean roots. Plant Physiol. 79, 494 - 498.

277. Widell, S., Lundborg, T. and Larsson, C. (1982) Plasma membranes from oat prepared by partition in an aqueous polymer two-phase system. On the light induced cytochrome b reduction as a marker for the plasma membrane. Plant Physiol. 70, 1429 - 1435.

278. Wieczovet, E., Lorenc-kubis, L. and Morawiecka, B. (1977) Purification and properties of acid phosphatase from Avena elatiol seeds. Acta Soc. Bot. Pol. 46, 481 - 488.

279. Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woodlock, J.J., (1969) Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. 30, 148 - 152.