STUDIES ON GLYCOPROTEIN ENZYMES OF PLANT STORAGE TISSUES : PURIFICATION AND CHARACTERIZATION OF ACID PHOSPHATASE ISOENZYMES FROM THE MEMBRANES OF PEANUT COTYLEDON CELLS

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Gratis.

DEDICATED

TO MY PARENTS

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, "STUDIES ON GLYCOPROTEIN ENZYMES OF PLANT STORAGE TISSUES : PURIFICATION AND CHARACTERIZATION OF ACID PHOSPHATASE ISOENZYMES FROM THE MEMBRANES OF PEANUT COTYLEDON CELLS" in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy in Department of Biosciences and Biotechnology, University of Roorkee, Roorkee, is an authentic record of my own work carried out during a period from Oct., 1986 to October, 1989, under the supervision of Professor C.B. Sharma.

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

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STUDIES ON GLYCOPROTEIN ENZYMES OF PLANT STORAGE TISSUES : PURIFICATION AND CHARACTERIZATION OF ACID PHOSPHATASE ISOENZYMES FROM THE MEMBRANES OF PEANUT COTYLEDON CELLS.

ABSTRACT

Highly enriched plasma membrane (PM), Golgi apparatus (GA) and endoplasmic reticulum (ER) fractions were obtained from the germinating cotyledons of peanut (Arachis hypogaea) and were identified by the presence of various marker enzymes. All the three membrane fractions were shown to contain the glycoprotein enzyme acid phosphatase (APase). The PM fraction exhibited the maximum activity of APase, followed by GA and ER, respectively. Since the microsomal membrane fraction of 2-days old cotyledons was found to be devoid of 5'-nucleotidase activity but possessing considerable amount of APase activity, it was used as the starting material for purifying APases present in the PM fraction. The isoenzymic pattern of APase in the PM fraction was found to change with progressive germination along with changes in the relative levels of the APase isoenzymes. The PM fraction of 0-days and 2-days old cotyledons were found to contain three distinct APase isoenzymes whereas 7-days germinated cotyledons contained only two APase isoenzymes.

APases were purified from the PM fraction by the selective solubilization of the enzyme in an active and stable form with 0.5% n-octylglucoside at a protein-to-detergent ratio of 2:3 in presence of Mg^{2+} and EDTA, followed by CM-Sephadex C-50 chromatography and gel filtration on Sephadex G-150 resin. The chromatographic step identified and separated three APase isoenzymes named APase I, APase II and APase III. Sodium dodecyl sulfate - polyacrylamide gel

electrophoresis (SDS-PAGE) established the homogeneity of the APase isoenzymes. The purification folds of APase I, APase II and APase III were 12.1, 10.6 and 9.9 while their respective molecular weights were 79 Kda, 76 Kda and 66 Kda. The PM-APases gave identical pH-activity profiles with a pH-optimum of 5.0. The isoenzymes were found to exist in monomeric forms at pH 5.0 consisting of single polypeptide chains, but they associated at pH 7.2 to give higher dimeric forms consisting of two polypeptide chains of identical molecular weight. The higher dimeric forms subsequently dissociated back into monomers on lowering the pH from 7.2 to 5.0.

The substrate specificities of the PM-APases was also found to be pH-dependent. At pH 7.2, the APase isoenzymes showed greater substrate specificity towards nucleotide - and sugar phosphates, whereas, at pH 5.0 all the three isoenzymes showed maximum specificity towards p-nitro phenyl phosphate (pNPP). The K_m and V_{max} values of APase I, APase II and APase III for the hydrolysis of pNPP were 0.43 mM, 0.50 mM and 0.48 mM and 2.1 x 10^2 µmol/min/mg, 2.8 x 10^2 µmol/min/mg and 2.4 x 10^2 µmol/min/mg, respectively. Orthophosphate inhibited APase I and APase II competitively but inhibited APase III non-competitively. Response of the PM-APases towards various cations and anions was found to be highly differential in nature.

Active sites of APase I, APase II and APase III were found to contain histidine residues. Cysteine residues also appeared to be involved with the active sites of APase I and APase II but not APase III. Mn and Zn were found to be associated with the PM-APases. The presence of Cu and Fe was not detected in any of the isoenzymes.

APase I, APase II and APase III contained 50%, 27% and 30% carbohydrate, respectively. Main sugars in case of APase I were D-man and GlcNAc while main

sugars in case of APase II and APase III were D-man and D-glu. The nature of peptide-carbohydrate linkage of APase I appeared to be N-glycosidic but O-glycosidic in case of APase II and APase III. Sodium metaperiodate strongly inhibited the activities of PM-APases.

The APase isoenzymes exhibited a broad range of thermal stability and temperature activity profiles. They also appeared to be quite stable for a considerable period of time when stored at -20°C.

Monensin brought about an increase in the level of GA-APase but a concomitant decrease in the level of PM-APase suggesting a blockage in intracellular transport of APases from GA to PM. Gibberellic acid (GA_3) treatment brought about an accumulation of APase activity in the PM but a decrease in APase activities in GA and ER fractions. It is suggested that APases present in the peanut cotyledon cells are intracellularly transported by the route ER $\rightarrow$ GA $\rightarrow$ PM.

Antibodies raised against peanut cotyledon PM-APases were seen to immunoprecipitate APase activities from ER and GA fractions as well. PM-APases were found to be immunologically related to each other. They were also found to be immunologically related to an APase isoenzyme (AP-I) present in the plasma membranes of pea cotyledons and APase present in the epididymis of ram and assumed to share some common antigenic determinants with them. It is suggested that APases from peanut cotyledon PM, pea cotyledon PM and ram epididymis share common epitopic regions and all of them may have evolved from a common ancestral gene.

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LIST OF ABBREVIATIONS

ADP	*Adenosine diphosphate*
AMP	*Adenosine-5'-monophosphate*
APase	*Acid phosphatase*
Asn	*Asparagine*
ATP	*Adenosine triphosphate*
CHAPS	*(3-[(3-Cholamidopropyl)-dimethylaminonio]. 1-propane sulfonate)*
CMP	*Cytidine monophosphate*
CTP	*Cytidine triphosphate*
ER	*Endoplasmic reticulum*
ER-APase	*Endoplasmic reticulum - acid phosphatase*
Fuc	*Fucose*
GA	*Golgi apparatus*
GA-APase	*Golgi apparatus - acid phosphatase*
Gal	*Galactose*
GDP	*Guanosine diphosphate*
GDP-Man	*Guanosine diphosphate - mannose*
Glc	*Glucose*
GlcNAc	*N-acetylglucosamine*
GMP	*Guanosine monophosphate*
GTP	*Guanosine triphosphate*
Glu-1-P	*Glucose - 1 - phosphate*
Glu-6-P	*Glucose - 6 - phosphate*
Gly-3-P	*Glyceraldehyde - 3 - phosphate*
K_m	*Michaelis-Menten constant*

Man	*Mannose*
pNPP	*para nitro phenyl phosphate*
Pi	*Inorganic phosphorus*
PM	*Plasma membrane*
PM-APase	*Plasma membrane - acid phosphatase*
SDS	*Sodium dodecylsulfate*
SDS-PAGE	*Sodium dodecyl sulfate - polyacrylamide gel electrophoresis*
Ser	*Serine*
Thr	*Threonine*
UDP	*Uridine diphosphate*
UDP-Glc	*Uridine diphosphate-glucose*
UMP	*Uridine monophosphate*
V_{max}	*Maximum velocity*
Xyl	*Xylose*

1.0 INTRODUCTION

Acid phosphatases (E.C.3.1.3.2) catalyze the hydrolysis of a variety of orthophosphate monoesters and are widely distributed in nature. In plants they have been localized in a wide variety of tissues and shown to be present in cell wall and various subcellular membranes (9,35,36,85,90,129,190). In general, plant acid phosphatases (APases) have been found to exist in multiple forms which vary considerably in their properties (90,95,129,152,213,182). Non-specific APases present in the cytosol have been known to be involved in the mobilization of stored phosphorus from germinating cotyledons to other anatomical parts of the plants for its early use and growth (71). Plant APases have also been implicated with the role of providing inorganic phosphate for metabolic, excretory and secretory purposes (152).

Although soluble cytoplasmic APases have been purified from a number of plants (35,63,95,152,199), the purification of plant membrane-bound APases have so far proved to be difficult and, therefore, they still remain poorly characterized. One of the reasons for this lapse has been the difficulty in preparation of various membrane fractions in pure form from plant cells since specific markers for different plant membrane fractions are not properly defined. The present investigation was undertaken with the purpose of characterizing the plasma membrane-bound APases from peanut cotyledon cells in an effort to bridge the gap of information regarding plant membrane-bound APases.

It is presently believed that the Golgi apparatus is involved in the biosynthesis, modification and intracellular transport of macromolecules in both plant and animal cells (177,193) and that it receives the biosynthetic

products from the endoplasmic reticulum (ER) which are destined for secretion or to become a part of the plasma membrane (19,145,189). Thus, the glycoproteins present on the external surface of the plasma membranes are presumably also constructed within the ER and must then be transported from the interior of the cell to the plasma membrane via Golgi apparatus. All along the route, the glycoproteins within vesicles are known to add glycosyl units and make other modifications. This process of intracellular transport of macromolecules, although well studied in animal cells, has not been properly studied in plant cells. Since APase was found to be present in the plasma membrane, Golgi and ER membrane fractions of peanut cotyledons, it was thought that APase present in the plasma membranes must be a useful glycoprotein for studying the process of intracellular transport in plant cells, which in turn would be useful in understanding the process of storage of seed proteins, which are mostly glycoproteins. Thus, the feasibility of using the plasma membrane-bound APase as a model glycoprotein enzyme for studying the intracellular transport in plant cells, particularly the storage cells, was also investigated.

The following were the main objectives that lay behind the present study:

1. To obtain highly enriched plasma membrane, Golgi apparatus and smooth endoplasmic reticulum fractions from germinating peanut cotyledons
2. To purify and characterize the APase isoenzymes present in the plasma membranes of peanut cotyledon cells
3. To study the immunological relationship of the plasma membrane-bound APase isoenzymes of peanut cotyledons with APases from other plant and animal sources
4. To investigate the possibility of using APase as a model glycoprotein for studying the process of intracellular transport and to establish

the route of intracellular transport of APase in peanut cotyledon cells.

It is hoped that the present study would help in a better understanding of the structural, functional and the evolutionary relationship between the various plasma membrane-bound APase isoenzymes. In addition, the results will also be useful in studying the intracellular transport of APases in cotyledon cells.

2.0 LITERATURE REVIEW

2.1 Plant acid phosphatases

2.1.1 Occurence and subcellular localization

Acid phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.2) are hydrolytic enzymes that catalyze the hydrolysis of a variety of orthophosphate esters and transphosphorylation reactions. They are widely distributed in nature and have been identified in a large variety of organisms and tissues. Acid phosphatases (APases) from plant tissues have been generally found to have a non-specific nature (152). Among various plant sources, APases have been reported to occur in pea cotyledons (57,90,139,199), sunflower seeds (152), maize scutellum (197), bean hypocotyls (129), barley seeds (9,10,93), soybean (60), corn (36), tomato fruit (35), rice grains (86,213), wheat grains (2), sweet potato tubers (201), rye (55), yam tubers (95), grasses (119), cellular slime mold (17), fungal myecelia (86,182), apricot kernels (86) and latex of laticifer plants (63).

Histochemically, APases have been extensively localized in cell walls from a number of plants. These include cell walls from pea cotyledons (90), bean hypocotyls (129), cultured rice plant cells (85), cultured tobacco cells (190), barley aleurones (9) and tomato fruit (35). APases have also been found to be present in the cytoplasm of corn cells (36), bean hypocotyl (129), yam tubers (95) and pea cotyledons (90); in the vacuoles of tobacco protoplasts (126) and red beet (110); in the Golgi and endoplasmic reticulum fractions of barley aleurone cells (93); in the microsomal fraction of bean hypocotyl

cells (129) and in the mitochondrial fraction of barley aleurones (93). The plasma membranes from corn roots (111) and corn leaf mesophyll protoplasts (154) have also been shown to exhibit APase activity. In onion cells, APase activities have been detected in and around the nucleolus, plasmalemma, dictyosomes and recitulum vesicles (130,175). Whole cell extracts of Phaseolus vulgaris contain several APase isoenzymes that are partially segregated subcellularly in the chloroplast, mitochondria and nucleus (144). Thus, APases seem to have an apparently ubiquitous subcellular distribution within plant cells.

2.1.2 Multiple forms

Plant APases often exhibit a tendency to occur in multiple forms and have been reported to occur in germinating peas (90), bean hypocotyl cells (129), yam tubers (95), tomato fruit (35), rice grain aleurones (213), wheat (2), rye (55), sunflower seed (152), barley (93), Aspergillus niger (182) and slime molds (17).

Six isoenzymes of violet-coloured APase have been isolated from cell wall preparations of rice plant cultured cells (85). All of them have a characteristic violet coloration and exhibit many different properties compared with the cytoplasmic isoenzymes (85). Sunflower seeds contain two forms of APase and it has been shown that these APase isoenzymes are specified by a single gene which has at least four codominant alleles (152). Fractionation of pea cotyledon extracts by gel filtration have confirmed the presence of

at least three isoenzymes of APase (isoenzymes I, II and III), of which there is little or no synthesis of isoenzyme I during germination (90). By contrast, isoenzyme II increases considerably in activity over the same period whereas isoenzyme III characteristically appears between day 5 and 6 following imbibition (90).

In yam tubers, two membrane-bound APases show small differences when compared to each other but show considerable differences in properties when compared with the cytoplasmic APase (95). Using starch gel electrophoresis, four isoenzymes of APase (AP-1, AP-2, AP-3 and AP-4) have been recognized in the aqueous extract of isolated aleurone particles from rice grains and all of them found to be glycoproteins (213). Of the four isoenzymes, AP-2 and AP-3 appear to be the products of enzymatic modification of AP-1 (213). Two main forms of APase have also been isolated from rye germs that are activated by several plant lectins (55).

APase represents one of the high abundance, developmentally regulated lysosomal enzyme in Dictyostelium discoideum. This enzyme exists in at least two isozymic forms, ap1 and ap2, during various developmental stages (17). The ap1 isozyme is present in vegetative cells and appears in both pre-spore and pre-stalk cells during all stages of development, whereas, ap2 isozyme represents the only known enzymatic marker of pre-stalk cells at the slug stage of development (17). A strain of Aspergillus niger (A. niger U20-2-5) was found to produce three forms of APase which differed in electrophoretic mobility and molecular weight (182). The significance of the existance of multiple forms of APases is still obscure although it is suggested (129) that there exists some functional assignment in the multiple forms of APase at least in case

of bean hypocotyl cells. Table I summarizes the occurence of multiple forms of APase from different sources.

2.1.3 Purification

Column chromatography with ion-exchange resins has been found to be a useful method for separation of APases by many investigators and consequently, has been the most widely utilized method for purification of APases, particularly from plant sources. APases from rye germ (55), bean (129), rice aleurone grain (213), soybean (60), sweet potato tuber (201), A. niger mycelia (182), rice plant cell wall (85), tomato fruit (35) and yam tuber (95) have been purified by various ion-exchange resins like CM-Sephadex, DEAE-Sephadex, CM-Cellulose and DEAE-Cellulose. APases from the endosperms of a major winter triticale have been recently purified to apparent homogeneity by employing a succession of DEAE-Sephacel, Con A-Sepharose 4B and Bio-gel P60 columns (36). Similarly, the APase from sunflower seeds was purified 1800-fold to homogeneity by chromatography on Con A-Sepharose, Affigel Blue Sepharose, DEAE-Sepharose and Sephacryl S-200 columns (152). An APase from the latex of Asclepias curassavica was purified by carboxymethyl-trisacryl and Sephadex G-150 columns (63) and a pea seed APase was purified 20-fold by application on a calcium-phosphate gel (199).

Many detergents have been used to solubilize bound-APases from the membranes and cell walls of many plant cells as an initial purification step. Triton X-100 has been used to solubilize APases from the cell wall of rice grains (85) and from the microsomes, mitochondria and cell walls of Phaseolus vulgaris (129). Ammonium sulfate precipitation (35,36,55,60,95,129,201,213)

TABLE-1

MULTIPLE FORMS OF APases FROM VARIOUS SOURCES

Source	No. of forms	Reference
Pea cotyledon	3	90
Phaseolus vulgaris		
i. Cell wall	2	129
ii. Mitochondria	3	129
iii. Microsomes	1	129
iv. Soluble fraction	12	129
Yam tuber	4	95
Tomato pericarp	2	35
Rice grain	20	213
Rye germ	2	55
Sunflower seed	2	152
Dictyostelium discoideum	2	17
Aspergillus niger	3	182

and acetone precipitation (182,201) are some of the other methods that have been extensively used to purify APases in initial stages.

2.1.4 Substrate specificity

Most of the APases generally exhibit a broad specificity towards orthophosphoric monoesters, the specificity being of nonspecific nature (36,63, 95,129,152,182,190). The APase from Asclepias curassavica latex hydrolyses many phosphorylated compounds but does exhibit some specificity since it does not hydrolyse UTP, β-glycerophosphate or glucose-6-phosphate (63). Two membrane-bound APases were found to be highly active towards phosphorylated sugars, particularly fructose 1,6-bisphosphate, unlike the cytoplasmic APase which had very little activity towards the same in case of yam tubers (95). It is thought that the high activity of the two membrane-bound APases with adenosine-5'-mono-, di- and tri-phosphates might yield the phosphate which is necessary for phosphorolysis reactions and also provide the energy necessary for transport mechanisms in which the enzymes may take part (95).

The APase from the shriveled seeds of a triticale exhibited more specificity towards glucose-1-phosphate (G-1-P) than the APase from the plump seeds of the triticale (36). This higher affinity may have significant consequences in situ as G-1-P is the substrate for ADP-Glc-PPase which provides ADP-glucose for the synthesis of starch for the filling of endosperm tissue. If G-1-P is depleted by the more active APase in shriveled seeds, the seed will be less filled than plump seeds (36).

2.1.5 General properties

2.1.5.1 pH-optimum and pH-stability

APases isolated from various plant sources generally exhibit pH-optima in the range of pH 4.0 - pH 6.0. In most cases pH-optima curves are relatively

broad (63,90,129,153,182,190), whereas, in some cases the optima are fairly sharp (95,201). The pH-optimum of APase in dormant maize scutellum is 5.2 which changes to pH 5.4 after 48 hours of seed germination (197). Interestingly, the pH-optimum of maize scutellum APase was found to be dislocated from pH 5.4 to pH 6.7 in presence of fluoride ions after 48 hours of germination although the electrophoretic mobility of the APase was unaffected (197). Values of optimum-pH and isoelectric points of some plant APases have been summarized in Table II.

2.1.5.2 Kinetic parameters

The K_m values of APases for the hydrolysis of paranitro phenyl phosphate (pNPP) vary greatly among various plant species as well as within the same species. For instance, in yam tubers the K_m values of membrane-bound APases were 2.9 and 3.1 mM (95), whereas, the sunflower seed APases had K_m values of 0.029 and 0.098 mM, respectively (152). Variations in K_m values exist among APase isoenzymes even within the same cell. For example, in Phaseolus vulgaris the APase isoenzyme present in the microsomes has a K_m value of 6.0×10^{-4} M, whereas, the APase isoenzyme present in the cell wall has a K_m of 1.8×10^{-3} M for the hydrolysis of pNPP (129).

Orthophosphate has been generally found to inhibit plant APases in a competitive manner (55,63,95,152,167,182,197) with widely ranging K_i values. In a winter triticale, phosphate was found to inhibit APase at 1 mM or more concentration in plump seeds unlike the shriveled seeds where phosphate stimulated APase at 1 to 6 mM concentration and inhibition observed only beyond this concentration (36). The P_i concentration which caused 50 percent inhibition of APase activity was 11 mM for plump seeds and 62 mM for shriveled seeds indicating a very high tolerance toward P_i concentration in the endosperm tissue

TABLE-II

OPTIMUM-pH AND ISOELECTRIC POINTS OF ACID PHOSPHATASES FROM VARIOUS PLANT SOURCES

Source	Optimum pH	Isoelectric point	Reference
Aspergillus niger (i form)	5.6	4.6	182
Rice cell wall			
i. Isoenzyme 1'	5.5	8.0	85
ii. Isoenzyme 2'	5.5	7.5	85
iii. Isoenzyme 3'a	5.5	7.2	85
iv. Isoenzyme 3'b	5.5	7.1	85
v. Isoenzyme 4'a	5.5	6.8	85
vi. Isoenzyme 4'b	5.5	6.7	85
Sweet potato tuber	5.8	5.0	201
Winter triticale	4.8	5.9	36
Tomato pericarp	5.5	-	35
Pea cotyledon			
i. Isoenzyme I	4.9	-	90
ii. Isoenzyme II	5.6	-	90
iii. Isoenzyme III	5.8	-	90

of the APase in shriveled seeds (36).

2.1.5.3 Effectors and Inhibitors

Plant APases are affected by various ions to varying degrees. Divalent ions like Zn^{2+}, Cu^{2+}, Mn^{2+}, Hg^{2+} and Mo^{2+} generally bring about an inhibition of APases (36,55,95,129,190), whereas, activation by Mg^{2+} and Ca^{2+} has been reported to occur in APases purified from the vacuoles of tobacco protoplasts (126) and both plump and shriveled seeds of triticale (36). Although, Zn^{2+} brings about a general inhibition, no effect of Zn^{2+} has been observed on the activity of membrane-bound APases in bean hypocotyls (129). A stimulatory effect of Mg^{2+} and a very important inhibition by EDTA of two membrane-bound APases in yam tuber has led to the suggestion that these two APases may be metalloproteins (95). F^- has been observed to greatly inhibit the activity of most of the APases (36,129,190,197). However, tomato fruit APase does not show any inhibition in presence of F^- ions (35). Effect of tartarate ions on APases range from inhibitory (55) to stimulatory (36) to noneffective (35). Hg^{2+} ions have been found to have a highly inhibitory effect on APases (36,55,95) and in some cases effecting a total inhibition of the enzyme activity.

2.1.5.4 Glycoprotein nature

Most of the plant APases are reported to be glycoproteins (36,55,60, 85,95,119,152,197,182,213). The glycoprotein nature of APases has been demonstrated by various methods such as binding to Con A (36,55,119,152,182,213), periodic acid-Schiff staining (60,85,95,182), anthrone method (36) and phenol-H_2SO_4 method (182,197). An Apase purified from the mycelia of A. niger has been found to contain 29 percent carbohydrate consisting of glucosamine, mannose and galactose (182). APases purified from both plump and shriveled seeds of

winter triticale are estimated to contain 12 percent carbohydrate (36). Two APases (APase B and APase C) from yam tuber seem to have almost the same proportions of hexoses and hexosamines which are slightly smaller than those found in the cytoplasmic APase (95). All these three APases were devoid of sialic acid (95). APases purified from maize scutellum both in dormant state and during the first 24 hours of germination have been found to contain 6 percent neutral sugars (197). Two isoenzymes (APase B_1 and APase B_2) purified from grass seeds are also reported to be glycoproteins containing D-mannose and D-glucose units (119). The violet-coloured APase of sweet potato tuber contains 8.8 - 9.8 percent neutral sugars mainly composed of fucose, xylose, arabinose, mannose and galactose (61).

The purified APase from rye germ extract is activated by several lectins and it is suggested that the carbohydrate part of the enzyme is engaged in the process of activation (55). Another report carries the suggestion that since APases from aleurone particles of rice seed are all glycoproteins, differences in their carbohydrate moieties may be responsible for the multiple forms of APase found in this system (213).

2.1.5.5 Immunological properties

In plants, immunological studies have been carried out on APases purified from wheat germ (3), tomato fruit (148), Dictyostelium discoideum (149), yam (96), grasses (133) and many other sources. While immunochemical differences have been found in APase isoenzymes from wheat germ (3) and tomato fruit (148), the cytoplasmic and membrane-bound APases from yam tuber (96) have been found to be immunologically related. Purified APases from all tissues of grass (independent of grass species) have shown cross-reactivity with monospecific antibodies raised against Poa pratensis seed APase B_1 indicating

the antigenic relationship between the APases of various grass species (133).

2.1.5.6 Other properties

Plant APases are not easily inactivated at elevated temperatures and are generally resistant to thermal inactivation around and above 50°C (35,85, 95,190). Violet-coloured APase isoenzymes present in the cell wall preparations of rice plant show relatively high thermostability and retain most of their activity upto 65°C (85). In tomato, one of the APase isoenzyme retains substantial activity at 60°C while the other isoenzyme retains 50 percent of its activity at 50°C (35). Three APase isoenzymes from yam tuber still show activity after 20 minutes at 50°C (95). In cultured tobacco cells, cell wall bound-APases retain 90 percent of their activity at 55°C even after 45 minutes, whereas, the solubilized APases retain only 50 percent activity under identical conditions (190).

APases have been seen to exhibit interesting behaviour when stored at different temperatures. Most of the APases show stability in activity when stored at -20°C for prolonged periods. However, freezing has been reported to cause inactivation of APase isoenzyme I in germinating peas when stored at -17°C, but not isoenzymes II and III which are relatively stable when stored at this temperature (90). APase activity in green tomatoes decreases rapidly when stored at 33°C but increases after tomatoes stored at 33°C for 10 days are transferred to 22°C (183).

Some plant APases are found to be associated with various metals as judged by absorption (60) and emission spectroscopic techniques (85,201). Purified APase from soybean was found to contain 0.42 µg manganese per mg dry weight of protein (60). Rice cell wall APases showed the presence of significant amounts

of calcium, magnesium and silicon (85). Metals like manganese, magnesium, boron and silicon were also detected in the purified APase preparation from sweet potato tubers (201). APases present in the membranes of yam tuber seem to contain magnesium and are reported to be probable metalloproteins (95). APases which have a characteristic violet colour have been generally found to be metalloproteins with manganese as the chief metal (60,85,201).

2.2 Plant membranes

2.2.1 General considerations

To study biochemical properties and functions of sub-cellular components, the general approach is to disrupt cells and separate various organelles and membranes. The isolated components or membranes are identified by their associated markers. Markers have been generally classified as morphological, biochemical and cytochemical (25,141,161). Morphological markers utilize the inherent morphology of some organelles to identify them in subcellular membrane fractions whereas the specificity of a cytochemical marker depends upon the presence of a unique constituent or constituents in a subcellular component (141). Hence, most of the cytochemical markers are biochemical in nature. Biochemical markers are usually intrinsic constituents of a given organelle or membrane, the most common being enzymatic markers. Enrichment in marker activity is taken to mean enrichment of the associated membranes (141,161).

The chemical composition of a membrane influences its functional properties including fluidity, permeability, protein activity, susceptibility to antibiotics and the structural configurations that the lipids assume under given thermal and ionic conditions (162). Many membrane processes like protein binding (196), insertion of newly synthesized proteins into membranes (209) and

host-pathogen interactions (41) may be influenced by the surface potential, i.e., by electrostatic interactions between the charged membrane surface and charged proteins. Biological membranes have been found to carry a net negative charge at neutral pH (99).

Most plant membrane isolation procedures have involved differential and sucrose gradient centrifugation (70,71) but a serious problem with this approach is cross-contamination of membrane fractions due to overlap in their densities (70). Free-flow electrophoresis (176), aqueous two-phase partitioning (99, 212,214) and isoelectric focusing (67) have been used to separate membranes according to differences in their surface charge properties.

2.2.2 Plasma membranes

2.2.2.1 Biochemical properties

The plasma membrane (PM) forms the outer border of plant cells which is in contact with the variable external medium. The outer surface of the PM is the first site of interaction between invading pathogens and plant cells and it plays an important role in resistance towards pathogens (99,107). In roots, the PM is responsible for the discrimination between and the uptake of ions from the soil solution (99). In addition, the PM is involved in several other important functions such as transport of ions and photosynthetic products, hormone-binding and responses and cell-wall synthesis (107). The PMs from plants as well as other organisms are dynamic, their composition varying with environment and development (210).

To date, comparatively little work has been done to elucidate the composition of PM of higher plants. One of the reasons has been the inability to prepare highly enriched fractions of PM from plants in quantities sufficient

for analysing the composition and subsequent characterization of PM. Whitman et al.(210) have, however, characterized the phospholipid composition of enriched PM fraction from developing soybean root with respect to both polar head groups and fatty acid composition.

Although many proteins of the PM are glycosylated, there is virtually no data on the carbohydrate moieties associated with this membrane. Recently Grimes et al. (68), after studying the carbohydrate composition of tomato PM, have found that protoplast-derived PM contains only mannose, galactose and glucose whereas tomato-fruit derived PM contains rhamnose, arabinose, xylose, mannose, galactose and glucose. 60 percent of the glucose was lipid-associated while total carbohydrate distribution was 43 percent lipid associated and 57 percent protein-associated in tomato fruit PM (68).

The PM surface in isolated vesicles is known to have binding sites for 1-N-naphthyl phthalamic acid, an inhibitor of auxin transport (198). PM from winter rye exhibits a tenfold greater binding specificity towards naphthyl phthalamic acid than the intracellular membranes and is not aggregated by $ZnCl_2$ unlike the intracellular membranes which are heavily aggregated by 10 mM $ZnCl_2$ (200). Thus, the PM seems to possess surface properties which are relatively different from those of the intracellular membranes. These differences in surface properties may in turn be related to the low protein content of the PM as compared to the intracellular membranes in plant cells (107).

2.2.2.2 Isolation procedures and markers

Density gradient centrifugation is at present the most commonly used method for PM isolation (70,107). Sucrose is the most widely used gradient material because it is inexpensive, transparent, highly soluble and does not interfere with most marker assays (141). In linear sucrose density gradients, the PM bands at a sucrose concentration equivalent to a density range of 1.14-1.17 g cm^{-3} (70,161). Less common gradient materials include serum albumin, ficoll

(112), sortibol, mannitol, dextran (57) and silica gel (122). A 20-50 percent sucrose range has been used for purification of PM from fractions obtained by differential centrifugation (20,70,123). PM has been successfully isolated from roots of corn by ficoll density gradient centrifugation (112).

As an alternative to gradient centrifugation, partition in aqueous dextran-polyethylene glycol two-phase systems was introduced for the purification of PM (121, 211). By this method membrane particles are separated according to their surface properties (4) rather than size and density. The PM vesicles obtained from plant material using phase partition are right side-out and mainly sealed (108). The PM preparations isolated by phase partitioning from various plant species and organs are highly purified (107,212) and have been shown to be practically free from contamination by intracellular membranes (107).

Other methods of PM isolation include free-flow electrophoresis (176) and isoelectric focusing (67) by making use of differences in surface-charge properties of PM. Although effective at fractionating PM, free-flow electrophoresis requires a major investment in equipment and is, therefore, not widely used (67). Isoelectric focusing on the other hand is relatively inexpensive but time consuming (176). Isoelectric focusing has been used to obtain small quantities of pure oat root PM apparently free of contamination by mitochondria or other membrane fractions (188).

No single marker has yet been found to be specific for plant PM and, hence, a combination of different markers is generally applied to ensure a high degree of purity of the membrane preparations (107). Phosphotungstate-chromate (PTA-CrO_3) stain has been widely used as a cytochemical marker for the PM <u>in situ</u> under defined conditions (172) even though the specificity of

the PTA-CrO_3 stain for the PM is clearly not absolute. This stain has, however, been used to provide tentative identification of PM vesicles in isolated subcellular fractions (161). The stain's selectivity *in situ* for the PM relative to the majority of other cellular membranes has now been documented for a variety of different plant tissues (142,172).

Mg^{2+} activated K^+ - ATPase has been suggested to be localized on the PM and correlations in the distribution of PTA - CrO_3 - positive vesicles and K^+-ATPase activity have been reported and interpreted as strong, direct evidence that this enzyme is PM - localized (82,112). Another enzyme glucan synthase II is also suggested to be primarily localized on the PM and correlated distributions of glucan synthase II and PTA-CrO_3-positive vesicles strongly support this suggestion (73,204). Anderson *et al*. (7) have presented convincing indirect evidence that *in vivo* glucan synthesis in pea cells occurs at the external surface of intact cells rather than on the internal membranes or the inner surface of the PM of damaged cells at the excision surface. However, convincing consistency regarding correlations in the (PTA-CrO_3) - (K^+-ATPase)-(glucan synthase II) pattern is lacking.

Several studies have employed Naphthylphthalamic acid (NPA)-binding as a marker for the PM (44,87,161,163) though independently - derived direct evidence that the binding activity is primarily PM - localized is lacking (161). It would seem, therefore, that the PTA-CrO_3 stain, when used cautiously (142) in combination with K^+-ATPase, glucan synthase II or NPA-binding, or ideally all three, can provide a positive marker package of some utility for tentative PM identification in membrane fractions (161).

2.2.3 **Golgi apparatus**

2.2.3.1 Biochemical properties

The Golgi apparatus (GA) is widely believed to be the site of synthesis

of cell wall matrix polysaccharides (134). Early evidence for the involvement of the GA in the production of polysaccharides includes cytochemical and biochemical observations. Data analogous to those obtained for polysaccharide synthesis indicate that the Golgi are involved in the synthesis and secretion of the cell wall protein extensin (37). It has further been confirmed that the GA is the site of synthesis of at least two cell wall matrix polysaccharides and that the GA is the source of the cell plate material deposited during cytokinesis in clover root cells (134).

The GA is believed to be a locus of dynamic, steady-state membrane differentiation where endoplasmic reticulum (ER) like membranes are progressively transformed into PM - like membranes by insertion or modification of constituents during migration across the dictyosome stack (23,26,135,136,138). A major aspect of the proposed dual membrane modification and secretory functions of the Golgi is the implied physical transfer of membrane constituents from ER to Golgi to PM (161). Evidences indicate that although the molecular constituents of the Golgi membranes overlap with those of ER and PM membranes (135,138), the molecular differences that underlie the obvious structural differences between these components have the potential to provide unique biochemical markers (161).

2.2.3.2 Isolation procedures and markers

Isolation of GA 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 (65). Two methods for the isolation of GA enriched fractions have been recently described (65). Differential centrifugation using different gradient systems comprise of the most widely used method of obtaining highly enriched Golgi fractions from plant cells. In sucrose density gradient, the GA bands at a sucrose

concentration of 29-33 percent which is equivalent to a density of 1.12 - 1.15 g cm^{-3} (65,161). Two fractions of GA 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 percent and 10-50 percent and centrifuging at 21,000 x g for 3 hours (5,6). Golgi fractions from corn coleoptiles have also been isolated by loading the 1000 x g supernatant on linear and non-linear sucrose density gradients (32,33,34).

GA consists of stacks of cisternae having localized swelling and isolated vesicles at the edges of cisternae thereby imparting a distinct morphology to this organelle. This typical morphology has been used for its identification during purification (131). Ionosine-di-phosphatase (IDPase) has been demonstrated to be biochemically associated with the GA (164) and a direct quantitative correlation between the distribution of IDPase activity and morphologically identifiable GA has been reported (73). The Golgi-associated IDPase is characterized by its latency (137,164) and thus distinguished from other activities. It is thus widely used as a Golgi marker (5,32,33,34).

Among the various glycosyl transferases detected in Golgi-rich fractions, glucan synthetase I is evidenced to provide a reasonable positive Golgi marker (161). The glucan synthetase I activity is expressed at low (μM) UDP-glucose and high Mg^{2+} (10 mM) concentrations and forms primarily β-1,4 linkages (161,164). Another enzyme galactosyl transferase (UDP-Galactose N-acetyl glucosamine galactosyl transferase) has been detected in Golgi fraction of onion stem (160) and recently used as a marker for Golgi fractions from suspension cultured cells of sycamore (5). Acid phosphatase (5) and α-mannosidase (6) have also been used as Golgi-markers from suspension cultured cells of sycamore. A peak of acid phosphatase has been found to coincide with latent IDPase activity

in sucrose gradient of pea epicotyls and suggested to be a marker of GA at least in pea (156).

2.2.4 **Endoplasmic reticulum**

2.2.4.1 Biochemical properties

Endoplasmic recitulum (ER) in plant cells forms an anastomosing system of smooth-surfaced (smooth ER, sER) or ribosomestuudded (rough ER, rER) membranes which are folded into a series of flattened sacs (cisternal ER, cER) or appear as a network of tubules (tubular ER, tER) (92). Direct evidences support a role for rER in the intracellular transport of proteins from rER to dictyosomes (74,75,94) and numerous interconnections between plant dictyosomes and ER suggest that protein transport can take different routes according to the type of proteins being sequenced (92).

During mitosis in corn and barley roots, disappearance of the nuclear envelope is coupled with the appearance of a network of cER that surrounds the spindle apparatus (92). This arrangement of ER seems to isolate the spindle from the cytoplasm and thus exclude cytoplasmic organelles. Elements of tER are also found interspersed among the dividing chromosomes of barley and corn (92). Tubular ER forms a complex network with vesicles during cell plate formation (76,79,80). These tubular elements are different from the vesicles that coalese to form the cell plate, and it has been proposed (92) that the tER acts as a template for the organization of the new cell wall. It has also been suggested that, by playing a role in Ca^{2+} sequestration, tER might also influence the fusion of vesicles in the cell plate and, therefore, cell wall synthesis (80).

Like other components of the endomembrane system, the ER of plant cells is composed largely of protein (60-65 percent of total membrane mass)

and lipid (30 percent) (155). Based on the qualitative differences in polypeptide composition, it has been suggested that in Ricinus communis, ER proteins from endosperm of maturing seeds are qualitatively different from the endosperm of germinating seedlings (92). Although there is a large body of evidence to suggest that the ER of plant cells is involved in the synthesis of proteins destined for transport to storage compartments or the cell exterior, the site of synthesis of ER membrane proteins in plant cells is not well understood (92).

Phospholipids are asymmetrically distributed in ER membranes and represent the major class of lipids of the ER membranes in plant tissues (92). In castor bean endosperm, 74 percent of total ER lipid is accounted for by phosphatidyl choline (33 percent), phosphatidyl ethanolamine (27 percent) and phosphatidyl inositol (14 percent) (45). The ER is regarded as one of the major sites of phospholipid biosynthesis in plant cells (132). Since the enzymes of phospholipid metabolism are concentrated in the ER, this membrane system may play a pivotal role in the biogenesis of other plant organelles (38).

2.2.4.2 Isolation procedures and markers

Density gradient centrifugation is the method of choice for the purification of ER membranes and sucrose is the medium that has been the favoured gradient material (92). Linear sucrose gradients that have been employed vary from 6 to 45 or 52 percent (206) and 15 or 16 to 48 or 50 percent (203). In step gradient, the ER is collected from the interface of 26/35 in a 4-step gradient of 35,26,23 and 13 percent (30) or the interface of 16/35 in a 2-step gradient (1,203). An ER-enriched fraction from Lepidium sativum roots has been recently prepared by loading the post mitochondrial fraction into a discontinuous sucrose gradient of 0.6, 1.0, 1.14 and 1.2 M (28,29).

Both rate-zonal and isopycnic density gradients have been utilized for the separation of ER from plant cells, and in cases where organelles have the same buoyant density but different size, a combination of both methods has been used to achieve purification of ER (91,97,163). Rate-zonal gradient centrifugation has been used to separate rough microsomes from mitochondria in barley aleurones since both band in a density range of 1.18-1.19 g cm^{-3} (91). Likewise, isopycnic centrifugation has been successfully used to separate microsomal membranes of corn coleoptiles that could not be separated by rate-zonal centrifugation (163). When microsomes were subjected to isopycnic centrifugation, ER and PM fractions were readily separated and were determined to have buoyant densities of 1.15 g cm^{-3} and 1.10 g cm^{-3}, respectively (163). A combination of rate-zonal and isopycnic centrifugation was used to separate ER from malate synthase-containing particles in homogenates from cucumber cotyledons (97).

When isolating ER, particular attention must be paid to the cation concentration and composition of the isolation medium. Plant ER has a high negative surface charge, and addition of cations, particularly divalent cations, to the isolation medium promotes aggregation of these membranes (92). The levels of Mg^{2+} and K^{+} in the medium must be rigorously controlled since these ions influence dissociation of ribosomes and their consequent detachment from the surface of the ER (174). The details of the various parameters of isolation medium like pH, osmolarity and temperature have been excellently reviewed in some recent reports (72,116,161,166).

Rough ER undergoes a shift in its buoyant density when the Mg^{2+} level of the homogenate is changed (161) and this Mg^{2+}-shift is the criterion that has received the widest acceptance for the identification of ER (116,161). In the absence of Mg^{2+}, ribosomes dissociate from the membrane resulting in

a decrease in its buoyant density from 1.15-1.19 g cm^{-3} to 1.10-1.13 g cm^{-3} (92,116,161). In barley aleurone cells, the ER membranes isolated in the presence of 3 mM Mg^{2+} have been found to be rough-surfaced possessing characteristic features of rER, while those membranes isolated in the absence of Mg^{2+} were devoid of ribosomes (92).

The NADPH - cytochrome c reductase activity appears to be exclusively confined to the ER (118) and has been used as a more reliable marker than NADH-cytochrome c reductase which has a multiple location (92,118,161) in plant cells. Choline phosphotransferase, an enzyme of phospholipid metabolism, has also been used by many investigators as an ER marker enzyme (92). One of the features which make enzymes of phospholipid metabolism particularly useful markers for ER is their location within the ER membranes. In particular, the terminal enzyme in phosphatidylcholine synthesis, CDP-choline : diglyceride transferase has been shown to have a distribution profile on sucrose gradients precisely coincident with that of NADPH-cytochrome c reductase and to respond in concert with the reductase in Mg^{2+} density shift experiments in case of castor bean endosperm (118). Combined with the accompanying microscopic evidence, there is little doubt that both these activities are essentially absolute markers of the ER in this system (118).

The binding of auxins to the ER is also a highly reliable feature which helps characterize the ER system in plants (92,163,173). Ray (163) has provided convincing evidence that NAA is bound specifically to the ER of Zea mays coleoptiles. Microsomes of plant and animal origin are known to possess a number of monooxygenase activities and evidence suggests (92) that these may be found primarily on the ER.

2.3 Plant glycoproteins

2.3.1 General considerations

Plant glycoproteins are covalent conjugates of proteins and carbohydrates in which the protein component is substituted by one or more heterosaccharides with a relatively low number (2-15) of sugar residues (104). It is now well known that the amino sugar D-glucosamine is a widespread component of plants and is usually found in combined form as a constituent of glycoproteins (89). Some of the glycoproteins secreted by maize endosperm cultures have terminal N-Acetylglucosamine residues (169). Other amino-sugars are rarely found in plants. The carbohydrate content of the storage glycoproteins is generally low (around 5 percent) and the main sugars are D-mannose and N-Acetyl-D-glucosamine (104). Other various sugars of plant glycoproteins are D-galactose, D-glucose, L-fucose, D-galactosamine, L-arabinose, D-xylose and D-mannosamine (104).

Higher plant glycoproteins include lectins, enzymes, reserve polymers, structural proteins, toxins and possibly primer molecules (179). Among higher plant enzymes, so far only a few have been conclusively shown to be glycoproteins. These include stem bromelain, ficin, horse radish peroxidases and some invertases (179).

A great variety of N-linked glycoproteins containing mannose and GLc NAc have been discovered in plants, particularly in seed storage materials (12,15), lectins (115,205), membrane components and secreted hydrolases or peroxidases (52,159). In addition, many and possibly all plants contain O-linked proteoglycans which may be structural wall components such as extensin or cell surface components such as arabinogalactan-proteins (104,105). The biosynthesis of such functionally interesting glycoproteins in plants is not fully understood,

particularly in growing tissues where cell surface and cell wall components are widely regarded as morphogenic determinants (157). Many N-linked plant glycoproteins contain high-mannose or modified oligosaccharides. The biosynthesis of these oligosaccharides involves the assembly of $Glc_3\ Man_9\ (GlcNAc)_2$-PP-dolichol, transfer of the oligosaccharide to protein and then assembly processing of the oligosaccharides (191). But little is known about these processing reactions.

Phytohemagglutinin (PHA) is unique among plant glycoproteins which have been studied in that it has one high mannose (Man_8) and one modified (fucosylated) oligosaccharide side-chain on each polypeptide (39). The high mannose chain is attached to Asn residue 12 and the modified chain to Asn residue 60 of PHA. Newly synthesized PHA in the rough ER has two high mannose (Man_9) chains. The modification of one of these chains in the Golgi requires the action of α-mannosidase and several glycosyltransferases (Glc NAc,Fuc,Xyl) (39).

Hydroxyproline-rich glycoproteins are substantial components of higher plant primary walls and are the sole components of Chlamydomonas walls (64).

The covalent attachment of carbohydrate to the peptide portion of glycoproteins usually involves the C-1 of the most internal sugar and the functional group of an amino acid in the peptide chain. The carbohydrate-peptide linkages in higher plant glycoproteins, which have been established with certainity, can be broadly classified into three groups (Fig.1) :

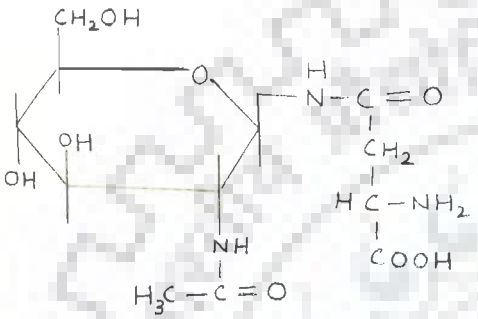
(A) N-Glycosidic Linkage : This is the glycosylamine bond involving N-acetyl-D-glucosamine and the amide group of asparagine (114).

(B) Alkali labile O-glycosidic linkage of D-galactose to serine (106).

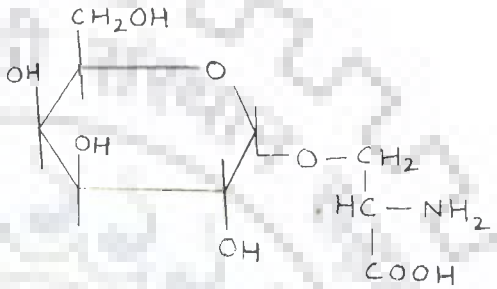
(C) Alkali stable O-glycosidic linkage involving L-arabinose and hydroxyproline (103).

FIGURE - 1

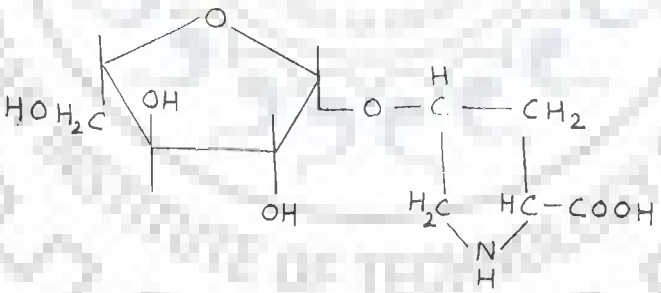
GLYCOPEPTIDE LINKAGES FOUND IN PLANT GLYCOPROTEINS



N-GLYCOSIDIC TO ASPARAGINE



O-GLYCOSIDIC TO SERINE (THREONINE)



O-GLYCOSIDIC TO HYDROXYPROLINE

2.3.2 Biosynthesis

The synthesis of glycoproteins start like other proteins on ribosomes. A specific amino acid region, the 'signal sequence' provides the attachment point of mRNA/ribosomes/nascent polypeptide complex to the membranes (18). This nascent polypeptide, led by 'signal sequence' passes to the cisternal space of the ER membrane compartment (113).

The first step in the synthesis of Asn-Glc NAc linked oligosaccharide is transfer of the core intermediate polyisoprenyl-pyrophosphoryl-Glc NAc_2-Man_9-Glc_3 to the endogenous proteins. Many plant membrane systems (12,49,51,52,187) are capable of assembling this typical lipid-linked oligosaccharide which appears to be the universal core intermediate for N-linked glycoprotein biosynthesis in animals and microorganisms (157). Although the transfer of oligosaccharides from this lipid to endogenous proteins was demonstrated with membrane-bound enzymes from soya roots (187), this product or its glucose-capped derivative accumulates and does not appear to be further metabolized in the crude pea systems (11). This could be due to a lack of suitable endogenous peptide acceptors in the system, unavailability of such acceptors at the site of glycolipid assembly or the site of the required <u>en bloc</u> glycosyltransferase,or inactivation of the latter enzyme (157). Other plant membrane systems have been shown (125) to transfer radioactivity from mannose-labeled lipids to added peptide acceptors which contain the requisite amino acid sequence, i.e. -Asp - X - (Ser/Thr) (98).

Lipid-linked oligosaccharides have to be present on the inner (lumen) surface of the ER in order for oligosaccharide transfer to nascent polypeptides to take place, and transmembrane glycosyl transfer must occur in the Golgi dictyosomes if they are to catalyze terminal glycoprotein processing (205). A recent

report (185) suggests that lipid-Glc NAc_2-Man_5 is indeed synthesized on the cytoplasmic side of the ER membrane and then translocated to the lumen where further mannose units and glucose are added. A similar set of reactions appears to take place in pea membranes (157) where an oligosaccharide equivalent to Glc NAc_2-Man_5 is the main product accumulated in charged glycolipids supplied with the appropriate sugar nucleotides (11).

Post translational processing of N-linked oligosaccharide that involves the removal of certain glycosyl residues as well as addition of new ones and initiation of O-linked oligosaccharides occur on Golgi-apparatus (177,193). Regulation and processing is controlled both by the specificity of glycosidases and the use of specific glycosyl transferase steps to trigger further glycosidase activities (192).

2.3.3 Physioloical functions

Although the function of the oligosaccharide chains of many glycoproteins is not yet established, there can be little doubt that in several instances their presence has an important biological significance. The surface of eukaryotic cells is decorated with a complex range of oligosaccharide chains, many of which are linked to protein by an N-glycosyl bond. The pattern of these chains changes during the biologically important transformation of cells associated with differentiation and in several cases interference with the pattern appears to effect a change in the transformation (78). Some glycosidases destined for lysosomes depend upon the presence of N-linked oligosaccharides which are subsequently phosphorylated to reach their subcellular site of action (78).

Plant membrane glycoproteins are mainly thought to be involved in recognition phenomenon (100,104) at the cell surface. They are also thought

to be involved in growth and differentiation of cells (104). A suggested function for glycosylation of proteins, as illustrated in yeast, is to make the protein very large and immobilize it outside the PM or in the cell wall (13). In higher plants too, immobilization of proteins, resulting in their definite localization in a tissue may be one of the roles of glycosylation (179). Several instances of the hydrophilicity of the oligosaccharide having a crucial effect on the conformation and/or orientation of the glycoprotein in a membrane have been described in the literature. In some cases, the hydrophilic mass has been shown to confer protection against proteolysis (78). Table III describes the effect of glycosylation on chemical and physical properties of some proteins.

2.4 Intracellular transport in plant cells

2.4.1 Biochemical aspects

The internal structure of a plant cell requires an elaborate system of energy transducing membranes to carry out photochemical reactions because of their ability to grow using sunlight as the only source of energy. Due to the complexity of these reactions and the underlying cytoarchitecture necessary to carry out these reactions, the problem of sorting, processing and transport of various proteins in plants is intrinsically more demanding than in other eukaryotic systems (43). Two models have been proposed to explain the transport events of membrane components and secretory products : The membrane flow model (133) and the membrane shuttle model (149). 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, ER, GA, PM, tonoplast and external envelopes of the chloroplast and mitochondria. In the membrane-shuttle model, each zone of the membrane system is viewed as a separate entity and communication between them is through vesicular package of information.

TABLE-III

EFFECT OF GLYCOSYLATION ON CHEMICAL AND PHYSICAL PROPERTIES OF PROTEINS[+]

Properties	*Effect of glycosylation*	*Protein*
Solubility	*Tends to enhance solubility in trichloroacetic acid*	*Arabinogalactan proteins, mucopolysaccharides*
Thermal Stability	*Increases*	*Asparaginase, Glucose oxidase, Invertase*
Resistance to proteases	*Increases*	*Extensin, RNase B, Plasma membrane glycoproteins, ATPase, Asparaginase*
SDS binding	*Increases*	*Glycophorin*

[+] *Details given in Ref. 104*

For many membrane proteins, the site of their primary insertion is not the site of their final functional residence. This appears to be the case with the integral membrane proteins of the Golgi complex, lysosomes and plasmalemma (151). The transport is effected by vesicular carriers proved to operate between the ER and the Golgi complex, in between different Golgi sub-compartments, between the Golgi complex and lysosomes, and between the Golgi complex and different domains of the PM (54). Vesicular transport is not limited to newly synthesized membrane proteins. Cells use it for the intracellular transport of secretory and lysosomal proteins (151), for exocytosis (149), endocytosis (77,124), transcytosis (171), as well as for membrane recycling (54, 150). There are indications that in certain cell types, secretory proteins and membrane proteins destined to the PM are transported by different vesicular carriers (69).

The mechanisms by which vesicular traffic is controlled are still not properly understood. However, it is established that vesicular transport involves membrane fusion-fission that leads to continuity between the lipid bilayer of the carrier and that of either the donor compartment or the receiving compartment at the two termini of the transport shuttle or circuit (150). It is assumed that the mechanisms that control vesicular traffic involve mutual recognition between signals and signal recognition complexes, as in the case of secretory and membrane proteins at the initial ER entry (151).

2.4.2 Intracellular targets

Five major targets of protein transport in plant cells have been recognized. These are the secretory pathway (ER,GA, vacuole and PM), the chloroplast, the mitochondria, the nucleus and the glyoxysome/peroxisome (43). Transport of proteins to these cellular compartments is mediated by amino terminal

'topogenic sequences'. For nuclear encoded-mitochondrial and chloroplast proteins, post translational targeting to the organelles involves recognition of a sequence of amino acids usually at the amino terminal end of the protein (43). These amino terminal TP (transit peptide) sequences are proteolytically removed after import into the organelle. Proteins imported by chloroplasts are further sorted into suborganelle compartments and recent evidences indiate that final targetting information is also present in the TP sequence (43).

Many of the vacuole proteins are synthesized in the rough ER and pass through the Golgi bodies. A few of these proteins (for example proteases and chitinase) are further exported from the vacuole to the PM (19). In some tissues, vacuoles differentiate to form 'protein bodies' which store proteins (19). The most thoroughly studied vacuolar proteins are the ones that accumulate in the 'protein bodies' of developing embryos. Two approaches are being taken to dissect the pathway(s) of vacuole-bound proteins (43). One approach is to express the genes in heterologous systems (nonplant) such as Xenopus oocytes and yeast, and the other is to express genes in transgenic plants. Recently, several research groups have reported the expression of genes encoding phaseolin and soybean 7S protein in petunia and tobacco plants and suggested that the signal sequences and glycosylation sites of seed storage proteins are conserved among different plant species (16,66,180).

Newly synthesized malate synthase has been demonstrated to be initially localized in the ER but found to be in the glyoxysome at later time intervals in plant cells suggesting that the membrane-bound enzyme is transferred from one compartment to another via a process of membrane flow (117). Catalase, a soluble enzyme found in microbodies, is synthesized on free cytoplasmic ribosomes (165). It is possible that the molecular assembly of glyoxysomes may involve

a combination of both cotranslational and post-translational segregation events (22).

The vast majority of chloroplast proteins that carry out complex biochemical processes are products of nuclear encoded genes (50) and as such they must be imported from their site of synthesis in the cytoplasm (43). Although the details of the actual import processes in chloroplasts are largely unknown, it is now clear that the outer envelope of the organelle plays an important role in precursor recognition (40). Recent experiments have shown that the TP (transit peptide) is both necessary and sufficient to target a foreign protein to the chloroplast compartment (27,84,178). Since cytosolic (120) and bacterial (42,207) proteins (which normally do not cross membranes) can be efficiently delivered to chloroplasts when presented as TP-fusions, it seems clear that organelle targetting is dependent only on the TP and the sequences surrounding the TP/mature junction (43).

2.4.3 Effect of Ionophores

It has been found that intracellular transport of secretory proteins is effectively slowed down by the action of certain ionophores (195). Several authors have attempted to distinguish distinct pathways of transport occurring within one and the same cell by making use of ionophores because the sensitivity of the intracellular transport of a given protein towards an ionophore is clearly diagnostic of the path the protein traverses (195). Monensin is a monovalent carboxylic ionophore that has been found to greatly retard the intracellular transport of several integral cell surface glycoproteins and some secretory proteins (24,195). Other carboxylic ionophores that are widely used include nigericin, dianemycin, X537A and A23187. Monensin assumes a roughly cyclic structure with the metal ion ringed by six liganding oxygens. Unlike nigericin, the carboxyl

group of monensin does not participate directly in complexation. Literature indicates that monensin inserts into lipid bilayers and can complex a given ion at one membrane interface and diffuse to the opposite face of the bilayer, where the ion can be released (195). For a return voyage, the ionophore may either carry another metal ion or become electroneutral by protonation of its carboxyl group. Such electroneutral transport is sensitive to the viscosity of the bilayer and is profoundly slowed at reduced temperature. Monensin-treated cells have greatly reduced K^+ ions and contracted mitochondria, suggesting extensive cation efflux, although in many cell types a number of biosynthetic activities proceed normally (195).

It has been possible experimentally to induce an alternative distribution of proteins in cotyledon cells (24). Treatment of jack beans and pea cells with monensin caused an accumulation of electron-dense deposits outside the PM. In jack beans, immunogold-labelling indicated the presence of ConA, α-mannosidase and the storage reserve canavalin in the deposits (24). This distribution was not observed normally, nor did it occur when the cotyledons were pre-treated with cycloheximide. These results have led to the inference that in cotyledon cells, monensin treatment induces a re-routing of newly synthesized products such that several products destined for the vacuole are all affected in an identical manner and are transported to the wronglocation (24).

Both monensin and nigericin, Na^+ or K^+/H^+ ionophores, cause swelling of mature cisternae of plant GA (21). Swelling is inhibited by the protonophore FCCP, by the inhibitor of lysosomal acidification, quercetin and by other agents known or suspected to inhibit 'proton pump' (21). It is thought that the monensin-induced swelling of GA cisternae may involve a proton gradient generating mechanism

but with properties unique to its subcellular location in specific portion of the GA (21).

2.4.4 Acid phosphatase as a model

Like several other secretory glycoproteins, APases have been found to be associated with various membranes during their transport. These include ER, vesicles derived from ER, proliferating ER, GA, central vacuole etc. (202). Gibberellic acid treatment has been found to increase the rate of hydrolase secretion from aleurone layers (93). Organelle isolation experiments and cytochemical data have also shown increased phosphatase activity in components of the endomembrane system having characteristics of ER and GA. Based on these observations it has been concluded that the ER and GA are involved in the synthesis and intracellular transport of APase in barley aleurone layers (93).

3.0 EXPERIMENTAL PROCEDURES

3.1 Materials

Tris, n-octyl-β-D-glucopyranoside, sodium dodecyl sulfate (SDS), CM-Sephadex C-50, Sephadex G-150, monensin and standard sugars such as D-glucose, N-acetylglucosamine, D-mannose and D-xylose were obtained from Sigma Chemical Co. (U.S.A.). Acrylamide, N,N'-methylene bis acrylamide, N,N,N',N'-tetramethyl ethylene diamine and β-mercaptoethanol were obtained from Serva (FRG). The protein standards for molecular weight determination were obtained from Bio-Rad (U.S.A.). Glucose-6-phosphate, glucose-1-phosphate, 5'-ribonucleotides and p-nitrophenyl phosphate used as substrates were purchased from Pierce Chemical Co. (U.S.A.). Complete and Incomplete Freund's adjuvants were from Difco Laboratories (U.S.A.). High purity analytical grade sucrose and agarose were purchased from Sisco Chemical Co. (India). All other chemicals used were reagent grade obtained from various commercial sources. Peanut seeds (large variety) were purchased from local seed stores and rabbits from local animal dealers.

3.2 Methods

3.2.1 Germination of seeds

Peanut seeds (Arachis hypogaea L.) were germinated on four layers of damp cheese cloth in plastic trays in a seed germinator under aseptic conditions. Surface sterilization of about 250 healthy seeds was carried out by treatment with 0.1% NaOCl for 5 minutes followed by thorough washing with water. The seeds were then transferred to plastic trays which were maintained at 35°C and 100% humidity in dark. During germination period the seeds were washed twice a day and infected seeds, if any, were removed carefully.

3.2.2 **Preparation of membrane fractions**

3.2.2.1 Crude membrane fraction

The crude microsomal membrane fraction was prepared by differential centrifugation as described by Bonner (20). Approximately 400 cotyledons (180-200 gm, fresh weight) of appropriate age were excised, washed and suspended in 400 ml of homogenizing buffer consisting of 50 mM Tris-HCl (pH 7.2), 0.25 M sucrose, 3 mM EDTA and 0.04% β-mercaptoethanol. Homogenization was performed by three 30 second bursts in a prechilled Waring blender and the homogenate filtered through 4-layers of cheese cloth. The filtrate was subsequently centrifuged at 12,000xg for 20 min followed by centrifugation at 105,000xg for 60 min. The 12,000xg to 105,000xg membrane pellet was suspended in homogenizing buffer and centrifuged at 105,000xg for 60 min. The membrane fraction thus obtained contained ER, GA and PM fractions and was termed as the crude microsomal membrane fraction. This fraction was suspended in 2 ml of 50 mM Tris-HCl buffer (pH 7.2) containing 0.04% β-mercaptoethanol and used for a number of preliminary experiments. Unless stated otherwise all operations were carried out at 0-4°C.

3.2.2.2 Pure plasma membrane fraction

The plasma membrane (PM) fraction was prepared essentially by the method of DuPont *et al*. (48) with slight modifications. For the separation of PM fraction from GA and ER fractions, the crude microsomal membrane fraction (12,000xg to 105,000xg pellet) was suspended in 4 ml of 25 mM Tris-HCl (pH 7.2) containing 20% (w/w) sucrose and 1 mM β-mercaptoethanol and layered carefully over 5 ml of 34% (w/w) sucrose prepared in the same buffer. This system was centrifuged at 80,000xg for 90 min. After centrifugation

the clear sucrose layers were removed carefully by aspiration. The microsomal membrane fraction and GA fraction remaining at the interface of 34/20% sucrose was removed with the help of a Pasteur pipette and the pellet containing the PM fraction (48) was suspended in 2 ml of the same buffer containing 20% (w/w) sucrose and 1 mM β-mercaptoethanol (Fig.2). The enzyme activities and protein content were determined immediately. The total protein recovered in the PM pellet varied between 16-20 mg per 100 gm fresh weight of cotyledons.

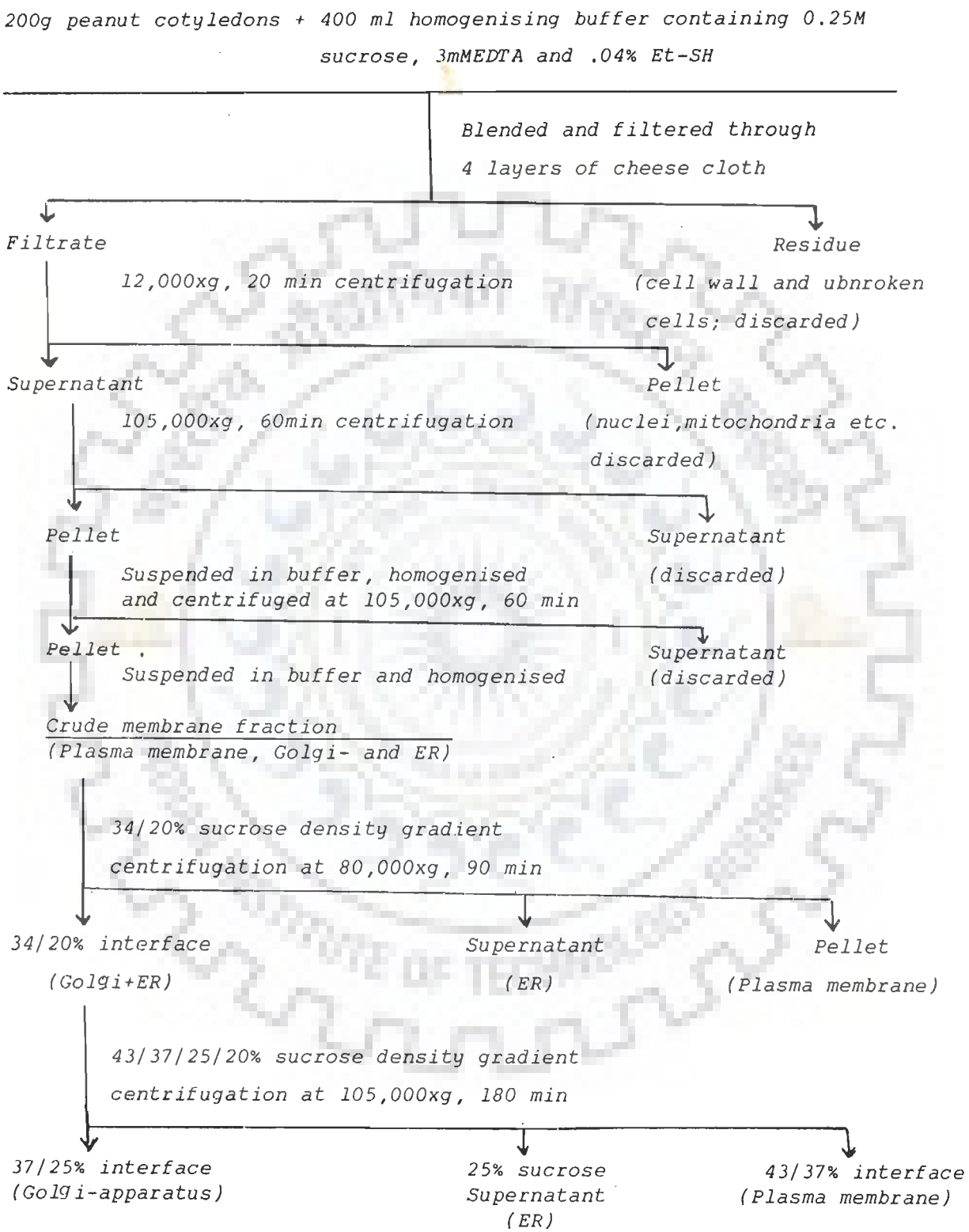
Alternatively, the PM vesicles were separated from other membranes by a discontinuous sucrose gradient centrifugation method as described by Hall (70). However, because of simplicity and ease of operation the method of DuPont et al. (48) was used to prepare highly enriched PM fractions from peanut cotyledons. The PM fractions were identified by the presence of the marker enzyme glucan synthetase II (70).

3.2.2.3Pure Golgi apparatus fraction

The plasma membranes were removed from the crude microsomal membrane fraction (12,000xg to 105,000xg pellet) by a step gradient of 34/20% (w/w) sucrose as described under 3.2.2.2. The membrane fraction staying at 34/20% interface was removed gently with the help of a Pasteur pipette and fractionated further into GA and ER fractions by the method described by Green (65) with slight modifications.

The 34/20% gradient interface membrane fraction was diluted with an equal volume of homogenizing buffer and centrifuged at 105,000xg for 60 min . The resulting pellet was suspended in 4 ml of 25 mM Tris-HCl (pH 7.2) containing 20% sucrose and 1 mM β-mercaptoethanol. This was carefully floated on a sucrose gradient system prepared by successively layering 7 ml of each

FIGURE - 2


200g peanut cotyledons + 400 ml homogenising buffer containing 0.25M sucrose, 3mMEDTA and .04% Et-SH
Blended and filtered through 4 layers of cheese cloth
Filtrate
Residue
(cell wall and ubnroken cells; discarded)
12,000xg, 20 min centrifugation
Supernatant
Pellet
(nuclei,mitochondria etc. discarded)
105,000xg, 60min centrifugation
Pellet
Supernatant
(discarded)
Suspended in buffer, homogenised and centrifuged at 105,000xg, 60 min
Pellet .
Supernatant
(discarded)
Suspended in buffer and homogenised
Crude membrane fraction
(Plasma membrane, Golgi- and ER)
34/20% sucrose density gradient centrifugation at 80,000xg, 90 min
34/20% interface
(Golgi+ER)
Supernatant
(ER)
Pellet
(Plasma membrane)
43/37/25/20% sucrose density gradient centrifugation at 105,000xg, 180 min
37/25% interface
(Golgi-apparatus)
25% sucrose
Supernatant
(ER)
43/37% interface
(Plasma membrane)

of 43%, 37% and 25% sucrose solution in the same buffer (Fig.2). This system was then immediately centrifuged at 105,000xg for 180 min. A major band appeared at 37/25% gradient interface which was removed carefully as before. This fraction represented the GA membrane fraction since the activity of the marker enzyme glucan synthetase I was found to be exclusively localized in this fraction. This fraction was used in all the experiments concerning the GA.

3.2.2.4 Endoplasmic reticulum fraction

The 25% supernatant part obtained after 43/37/25/20% sucrose density gradient centrifugation during GA purification (3.2.2.3) was removed carefully and diluted with an equal volume of 25 mM Tris-HCl (pH 7.2) and centrifuged at 105,000xg for 60 min. The pellet obtained was suspended in homogenizing buffer and used as enriched ER membrane fraction (Fig.2). The enrichment fold was determined by assaying the mannosyl transferase activity in different membrane fractions, relative to enzyme activity present in the crude microsomal membrane fraction.

3.2.3 Solubilization of membrane-bound acid phosphatases

All steps of solubilization and purification were carried out at 0-4°C. The membrane fraction (112 mg protein) was suspended in 10 ml of 50 mM Tris-HCl (pH 7.2) containing 0.25 M sucrose, 0.04% (v/v) β-mercaptoethanol, 20 mM $MgCl_2$, 5 mM EDTA and 0.5% of the nonionic detergent n-octyl-β-D-glucopyranoside at a protein-to-detergent ratio of 2:3. This suspension was gently stirred on a Vortex mixture for 2 min followed by three strokes in a Teflon homogenizer. The homogenate was immediately centrifuged at 105,000xg for 60 min and the supernatant used as solubilized enzyme for further purification and analysis.

3.2.4 CM-Sephadex C-50 column chromatography

The solubilized enzyme was dialyzed 16 h against 100 volumes of 50 mM sodium acetate buffer (pH 5.0). The dialyzed enzyme (20 mg protein) was then loaded on a CM-Sephadex C-50 column (1.5 x 20 cm) which was preequilibrated with the same buffer. The column was washed with equilibration buffer (twice the bed volume) to remove the unabsorbed proteins. The absorbed proteins were eluted by a linear gradient of 0-500 mM NaCl using a single mixing container with 50 ml of 50 mM sodium acetate buffer (pH 5.0) and a reservoir with 50 ml of the same buffer containing 500 mM NaCl. One ml fractions were collected at a flow rate of 0.2 ml/min and aliquots (0.2 ml) from every other fraction were analyzed for the enzyme activity and protein content. Top few fractions of each peak were pooled and concentrated by ultrafiltration.

3.2.5 Sephadex G-150 column chromatography

Sephadex G-150 columns (0.9 x 25 cm) were preequilibrated with either 50 mM sodium acetate buffer (pH 5.0) or 10 mM Tris-HCl buffer (pH 7.2) for a sufficient period of time. The enzyme fractions obtained after CM-Sephadex C-50 column chromatography were dialyzed overnight against 100 volumes of the equilibration buffer and loaded on to Sephadex G-150 columns. One ml fractions were collected at a flow rate of 1 ml/15 min and aliquots (0.2 ml) from every other fraction were analyzed for enzyme activity and protein content.

3.2.6 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in presence of sodium dodecyl sulfate (SDS) was carried out on 10% gels in cylindrical tubes (0.5x 8.0 cm) by procedures described by both Weber and Osborn (208) and Laemmli

(101).

In case of SDS-PAGE performed by the method of Weber and Osborn (208, 158), sample buffer contained 0.01 M sodium phosphate (pH 7.0), 1% SDS and 5% β-mercaptoethanol; gel buffer contained 0.1 M sodium phosphate (pH 7.0) and 0.2% SDS and electrophoresis buffer contained 0.05 M sodium phosphate (pH 7.0) and 0.1% SDS. When SDS-PAGE was carried out using a modified Laemmli's discontinuous buffer system (101,158), sample buffer contained 0.01 M Tris-HCl (pH 8.0), 1% SDS and 5% β-mercaptoethanol; gel buffer contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS while the electrophoresis buffer contained 0.1% SDS, 0.05 M Tris and 0.384 M glycine (pH 8.3).

The protein samples (40-100 µg) were first heated for 5 min at 100°C and layered through the electrophoresis buffer in 40% sucrose solution. Reference proteins used for molecular weight determination were also subjected to the same treatment. Bromophenol blue was used as the tracker dye. The gels were run at a constant current of 8 mA/tube at room temperature and the protein bands located by staining in 0.5% Coomassie Brillian blue prepared in a solution of water : methanol: acetic acid in the ratio of 50:45:5 (v/v/v) for 6 h. The gels were destained and stored in 7% acetic acid.

Polyacrylamide gel electrophoresis without SDS and β-mercaptoethanol were performed similar to SDS-PAGE. Staining and destaining procedures were also identical to that of SDS-PAGE.

*3.2.7 **Analysis of sugar moieties of glycoprotein enzymes***

3.2.7.1 Removal of N-glycosidically linked oligosaccharide from the glycoprotein enzyme

The glycoprotein sample (400 µg protein) was treated with 1.5-5.0 units of N-acetyl-β-D-glucosaminidase (Sigma Chemicals Co.) and incubated

at 37°C for 24 h. The reaction was stopped by placing the reaction vessel in a boiling water bath for 10 min followed by the addition of 10% trichloroacetic acid (TCA). After cooling the denatured protein precipitate was separated from the supernatant part by a 60 min centrifugation at 50,000xg. The precipitate was washed twice with 7% TCA and the supernatants pooled. The total carbohydrate content in both precipate and supernatant were determined. The decrease in the carbohydrate content of the acid precipitated protein and a corresponding increase in the carbohydrate content of the supernatant indicated the removal of the carbohydrate by endo-N-acetyl-β-D-glucosaminidase treatment.

3.2.7.2 Release of neutral sugars and hexosamine

For the release of neutral sugars, the method of Spiro (186) was followed. 0.2 ml of the glycoprotein sample (400μg protein) was hydrolyzed in 1 ml of 4N HCl for 6 h in sealed tube at 100°C. After acid hydrolysis the hydrolysate was evaporated to dryness under a stream of nitrogen repeatedly to remove the residual HCl. The resultant residue was dissolved in 5 ml of distilled water and passed through a column packed with mixed bed resin of Dowex-50 (H^+ form) and Dowex-1 (Cl^- form). The eluent was collected in a clean glass vessel. The column was then washed two times with 10 ml of distilled water and the combined filtrate evaporated to dryness in vacuo. This fraction represented the neutral sugar fraction.

The hexosamine fraction was obtained by eluting the mixed resin column of Dowex-50 (H^+) and Dowex-1 (Cl^-) with 25 ml of 2N HCl after the elution of the neutral sugars. This fraction was also evaporated to dryness in vacuo as before.

3.2.7.3 Analysis of monosaccharide by HPLC

Analysis of the released neutral sugars and hexosamine was performed by high performance liquid chromatography carried out with Shimadzu LC-4A HPLC equipped with a UV spectrophotometric detector SPD-2AS and a chromatopac C-R2AX data processing system. The sugar samples for HPLC were derivatized to alditol acetate derivatives by the method of Spiro (186) with slight modifications as described below.

The dried sugar samples after the acid hydrolysis step were dissolved in methanol and 0.2 ml (25-50 µg) transferred into acid washed glass vials. These were dried under a stream of nitrogen and washed two times with 0.5 ml of methanol. After drying the sugar samples were subsequently reduced by adding 1 ml of 0.25 M freshly prepared sodium borohydride solution and left overnight at 4°C. The reduced sugars were dried and washed three times with 1.5 ml of methanol to remove all traces of boric acid.

Acetylation was carried out by the additon of 0.25 ml of pyridine and 0.25 ml of acetic anhydride to dry samples in sealed tubes at 100°C for 1 h. After acetylation the samples were washed twice with 0.25 ml pyridine followed by two washings with 0.2 ml acetonitrile (CH_3CN). The samples were finally suspended in 1 ml of CH_3CN for injecting into the HPLC column. Standard sugar samples were also derivatized in an identical fashion.

Sample (5 µl) was injected to a 25 cm, 5 µm Zorbax C-18 column. The column was eluted with a mixture of CH_3CN: H_2O in a ratio of 80:20 for 15 min at a flow rate of 0.8 ml/min. The sugar peaks were detected by the UV spectrophotometric detector at 250 nm. The monosaccharides of the glycoprotein enzymes were identified by comparing their retention times with those of standard sugars.

3.2.8 **Enzyme assays**

3.2.8.1 Acid phosphatase

Acid phosphatase activity was measured by a slightly modified method of Odds and Hierholzer (146) using p-nitrophenyl phosphate as substrate. The incubation mixture (1 ml) contained 50-100 µg enzyme protein, 2.0 mM p-nitrophenyl phosphate and 50 mM acetate buffer (pH 5.0). The reaction was terminated after 15 min incubation at 30°C by the addition of 1.5 ml of 4% Na_2CO_3. The absorbance of yellow color of p-nitrophenyl released was measured at 420 nm against the control to which enzyme had been added after termination of the reaction. Specific activity was expressed as µmole p-nitrophenol produced per minute per milligram protein.

3.2.8.2 5'-nucleotidase

5'-nucleotidase activity was measured with 5'-adenosine monophosphate (5'-AMP) as substrate as described by Riemer and Widnell (170) with slight modifications. The standard reaction mixture contained 1-2.5mM 5'-AMP, 50 mM sodium acetate buffer (pH 5.0) and 0.1 ml of the enzyme preparation (8-70 µg protein, depending on the form of enzyme assayed) which was added last, in a total volume of 1.0 ml; control incubations contained no substrate. Incubation was carried out at 30°C for 15 min and the enzyme activity terminated by adding 0.5 ml of cold 20% trichloroacetic acid. Protein was removed by centrifugation and Pi was determined in the supernatant fluid by the procedure of Fiske and Subba Row (56). The specific activity of 5'-nucleotidase was expressed as µmoles of Pi liberated by dephosphorylation of 5'-AMP per min per milligram protein under assay conditions.

3.2.8.3 Glucan synthetase I

Glucan synthetase I activity was measured as described by Green (65) using low concentration of UDP-glucose and 10-20 mM Mg^{2+}. The reaction

mixture contained 100 µg membrane protein, 100 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 10 mM cellobiose, 4 mM EDTA, 2 mM β-mercaptoethanol and 0.1 µCi of UDP-[^{14}C]-glucose (6 nmol) in a total volume of 0.1 ml. The reaction was terminated by heating the mixture to 95°C followed by the addition of 10 mg of powdered cellulose as carrier for newly synthesized β-glucans. The precipitate was collected by centrifugation and washed sequentially three times (3X) with 1 ml of hot glass distilled water, 1X with $CHCl_3/CH_3OH$ (1:2, v/v), 1X with methanol and 2X with 1N NaOH. The alkali insoluble residue was rinsed with 1 ml of distilled water and the radioactivity measured by suspending in scintillation fluid (dioxane cocktail containing 10% naphthalene and 0.5% PPO in dioxane) and reading in Beckman LS-1801 liquid scintillation counter.

3.2.8.4 Glucan synthetase II assay

Glucan synthetase II activity was measured as described by Ray (163) using high concentration of UDP-glucose in the absence of Mg^{2+}. Membrane fractions (100 µg protein) were incubated in 0.1 ml of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.1 µCi of UDP-[^{14}C]-glucose (260 Ci/mol) and 0.5 mM unlabelled UDP-glucose at 25°C for 20 min. The reaction was terminated by the addition of 1 ml of 70% (v/v) ethanol, 50 µl of 50 mM $MgCl_2$ and 50 µl of boiled plasma membranes (0.2 to 0.3 mg protein) as carrier for the labelled products. The mixture was immediately boiled for 1 min and after standing overnight at 4°C, was centrifuged at 1500xg for 5 min. Precipitated particulate material was washed 4X with 70% ethanol to remove all unreacted radioactive substrate and ethanol soluble byproducts. The washed precipitate was suspended in scintillation fluid and radioactivity measured in Beckman LS-1801 liquid scintillation counter.

3.2.8.5 Mannosyl transferase

The method of Lehle et al. (109) was used to measure the mannosyl transfer from GDP-[^{14}C]-mannose to endogenous as well as exogenous lipid acceptor (dolichol phosphate). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 0.1 µCi GDP-[^{14}C]-mannose. 100-200 µg protein (particulate membrane fraction) was incubated with the reaction mixture in a total volume of 70 µl. For the incorporation of mannose to the exogenous lipid acceptor, 10 µl of dolichol monophosphate (10 µg) was mixed with 10 µl of 0.1 M Mg-EDTA and dried under nitrogen. The dried lipid was dispersed with 10 µl of 5% nonidet. This lipid was then incubated with the reaction mixture as described above. The reaction was stopped after 30 min 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. Upper aqueous phase was discarded and the lipid phase was washed by the method of Folch et al. (58). The washed lipid was dried in a vial and the radioactivity measured after suspending the dried material in scintillation fluid (dioxane cocktail). Blank was prepared under identical conditions and the enzyme protein was added after termination of the reaction.

3.2.8.6 Glucose-6-phosphatase

The activity of glucose-6-phosphatase was assayed as described by Aronson (8). The reaction mixture contained 1 mM sodium acetate buffer (pH 5.0), 10 mM glucose-6-phosphate and 0.1 ml (50-100 µg protein) of membrane protein. The reaction mixture was incubated at 35°C for 15 min. The liberated Pi was determined by Fiske and Subba Row method (56).

3.2.9 Protein estimation

Protein was estimated by the method of Lowry et al. (53) with slight modifications using bovine serum albumin as standard. β-mercaptoethanol was removed by heating the protein samples at 60°C for 30-60 min in a water bath before performing the Lowry method (194). Since the solubilized enzyme contained 0.5% n-octylglucoside, all samples (including standard proteins) were normalized with regard to detergent and the estimation was done in the presence of 0.1% SDS (47).

3.2.10 Determination of optimum pH

A fixed amount of enzyme protein (50 µg) was incubated with 2 mM p-nitrophenyl phosphate at 30°C for 15 min in suitable buffers of varying pH. The buffers used were 50 mM sodium acetate buffer (pH 3.5-5.5) and 50 mM Tris-HCl (pH 6.0 to 9.5). Control assays at each pH were run simultaneously. Enzyme activities were represented as percentage of maximum activity by plotting pH versus % maximum activity.

3.2.11 Determination of kinetic parameters

Lineweaver-Burk plots were plotted in order to determine the values of apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of the glycoprotein enzymes. Enzyme assays were carried out in a normal manner by using varying amounts of p-nitrophenyl phosphate (0.25 - 2.0 mM) in 50 mM sodium acetate buffer (pH 5.0). Care was taken that levels of substrate concentration used were such so as to give from partially to fully saturated enzyme system. Linear plots were obtained by plotting reciprocals of substrate concentration (1/S) and velocity (1/V). The intercepts on X-axis and Y-axis were equal to $-1/K_m$ and $1/V_{max}$ respectively.

Inhibitor constant (K_i) and the type of inhibition of the enzymes

was calculated by plotting Lineweaver-Burk plots in the presence of a fixed concentration of inhibitor(0.5 mM). Intercept on the X-axis was equal to $-1/K_i$.

3.2.12 Production of monospecific antibodies against purified glycoprotein enzymes

Monospecific polyclonal antibodies (Abs) were raised against purified membrane glycoprotein isoenzymes in rabbits by following a predetermined immunization schedule.

3.2.12.1 Antigen preparation

Purified glycoprotein samples were dialyzed extensively against 50 mM sodium acetate buffer (pH 5.0) and concentrated by ultrafiltration to a protein concentration of 500 µg/ml. 300 µg of each sample (0.6 ml) was emulsified with an equal volume (0.6 ml) of Complete Freund's adjuvant by repeatedly passing the emulsion through a 17G needle attached to a 2 ml syringe till the mixture turned into a thick while uniform suspension. Before injecting the homogeneity of this preparation was tested by gently floating a drop of it in a beaker of water. The compactness of the drop over the water surface indicated a good homogeneous preparation.

3.2.12.1.1 Purification of pea cotyledon PM-APases

The PM fraction from pea cotyledons was prepared similar to the PM fraction from peanut cotyledons as described under 3.2.2.2. The homogenizing buffer consisted of 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose and 5 mM β-mercaptoethanol. The PM-bound APases were solubilized using 1% CHAPS in presence of 5 mM EDTA at a protein-to-detergent ratio of 2:3 as described under section 3.2.3. The solubilized PM fraction was dialyzed overnight against 100 volumes of 10 mM Tris-HCl (pH 7.4) and loaded on a DEAE-Sephadex A-50 column (1.5x20cm) which was preequilibrated with the same buffer. The absorbed proteins were

eluted by a linear gradient of 0-250 mM NaCl using a single mixing container with 50 ml of 10 mM Tris-HCl (pH 7.4) and a reservoir with 50 ml of the same buffer containing 250 mM NaCl. 1 ml fractions were collected at a flow rate of 0.2 ml/min. Two APase activity containing peaks eluted from the DEAE-Sephadex A-50 column at 0 mM (washing) and 78 mM NaCl respectively. These were termed as AP-I (minor peak) and AP-II (major peak). The AP-II peak was further purified on CM-Sephadex C-50 column as described under secton 3.2.4. The AP-II eluted from CM-Sephadex column at 100 mM NaCl. The AP-I and AP-II fractions were extensively dialyzed against 50 mM sodium acetate buffer (pH 5.0) to remove all traces of salt and brought to a concentration of 500 µg/ml by ultrafiltration.

3.2.12.1.2 Partial purification of Ram epididymal APase

100 mg of Ram epididymal tissue was homogenized with 2 ml of 0.1 M sodium acetate buffer (pH 5.0) to a fine slurry in the presence of acid-washed sand; the mixture was centrifuged at 1500 r.p.m. for 10 min to remove sand and other precipitated matter. The supernatant was found to contain considerable amount of APase activity which was partially purified by 30-60% ammonium sulfate precipitation followed by extensive dialysis against 50 mM sodium acetate buffer (pH 5.0). This partially purified APase fraction was also brought to a concentration of 500 µg/ml.

3.2.12.2 Immunization schedule

1 ml of each of emulsified glycoprotein was injected subcutaneously into different rabbits. The injections were given on the dorsal side of the animals at multiple sites. After the first injection, subsequent injections were prepared by emulsifying the purified glycoproteins with Incomplete Freund's adjuvant and administered at weekly intervals over a period of six weeks.

3.2.12.3 Collection of antisera

Six weeks after the first injection, the rabbits were bled by the ear vein and around 2 ml of blood collected from each animalin conical tubes. Immediately after collection of blood, the tubes were kept in an inclined position at 37°C for 60 min till a straw coloured fluid was seen to appear over the clot. The tubes were then centrifuged at 1000 r.p.m. for 10 min and the antisera gently decanted into stoppered glass vials and stored at -20°C. The tubes were kept at 4°C overnight for the extraction of the remaining antisera. The presence of antibodies in the antisera was detected by both immunodiffusion and immunoprecipitation.

3.2.12.4 ***Detection of antibodies***

3.2.12.4.1 Immunodiffusion

Immunodiffusions were performed in 1% agarose gels containing 0.9% NaCl (w/v) by the method of Ouchterlony (147). 4 ml of melted agarose were poured on clean glass slides (7.5 x 2.5 cm) which were kept over a flat surface and allowed to solidify at room temperature so that gels of 1.5 mm thickness were formed. A 10 µl well was punched out from the centre of the agarose gel and 4 wells of 8 µl capacity were punched out in a circular fashion around the central well at an equidistance of 1 cm from it. The central well was filled with 10 µl of the purified glycoprotein (10 µg protein) and three surrounding wells with the corresponding antisera at different dilutions. The fourth well was filled with preimmune sera and served as control. The loaded gels were kept overnight at 37°C in a humid chamber. The appearance of white precipitin bands between the central well and the surrounding wells indicated the presence of antibodies in the antisera. No precipitin band was found to appear between the central and the control well. The precipitin bands were stained with 1% Coomassie Brilliant blue for 30 min and destained in 10% acetic acid.

3.2.12.4.2 Immunoprecipitation

0.2 ml (100 µg protein) of each of the purified glycoprotein isoenzyme was added to an equal volume of its corresponding antisera in conical test tubes (0.8 x 8 cm). The tubes were vortexed for 20 seconds at low speed and left undisturbed at 4°C for 7 days. The appearance of a white precipitate at the bottom of the tubes indicated the presence of antibodies in the antisera. Tubes containing purified glycoprotein isoenzymes without their corresponding antisera were used as controls. Yet another set of controls was prepared by adding preimmune sera (instead of antisera) to the purified isoenzymes. Control tubes did not show any precipitation.

For determining the amount of immunoprecipitation, the tubes were centrifuged at 1500 r.p.m. for 5 min and the enzyme activity (APase activity) in the supernatant fluids measured by taking 0.2 ml aliquots. The degree of immunoprecipitation was calculated in terms of percentage residual activity remaining in the supernatant with respect to the control whose percent residual activity was considered to be 100%.

4.0 *RESULTS*

4.1 *Preparation of membrane fractions*

Fig. 2 shows the flow chart for the preparation of PM, GA and ER membrane fractions from peanut cotyledons. The cell free extract was prepared in a medium containing 0.25 M sucrose, 3 mM EDTA and 0.04% β-mercaptoethanol without $MgCl_2$. Under these conditions the ribosomes remained detached from the rough ER and the integrity of PM as well as other organelles was maintained (65,70). The centrifugation of the cell free extract at 12,000xg removed mitochondria, nuclei, cell wall debris etc. This fraction was discarded. The supernatant when centrifuged at 105,000xg for 60 min resulted in a crude membrane pellet mainly consisting of PM, GA and ER. The supernatant (cytosol fraction) was discarded.

The PM fraction was separated from the GA and ER by involving a two-step sucrose gradient (34/20%, w/w) in 25 mM Tris-HCl (pH 7.2) containing 1 mM β-mercaptoethanol. On centrifugation at 80,000xg for 90 min, the crude membrane fraction (12,000 - 105,000xg pellet) was fractionated into three clear bands; (i) the pellet representing the PM (ii) A band at 34/20% interface representing mainly GA and ER with some contamination of PM, and (iii) a diffused band near the top containing ER and lipids.

These bands were carefully separated. The 34/20% interface band was subjected to a step-wise sucrose gradient (43/37/25/20%, w/w) centrifugation. A major membrane band at the 37/25% interface represented the Golgi fraction. The supernatant above the 37/25% interface containing mostly ER, was removed, adjusted to a sucrose concentration of 12% and centrifuged at 105,000xg for 120 min. The pellet formed represented the ER fraction.

4.2 Distribution of marker enzymes in various membrane fractions of *Arachis hypogaea* cotyledons

The distribution pattern of various marker enzymes for the identification of PM, GA and ER membrane fractions of peanut cotyledon cells has been summarized in Table IV. Results indicate that approximately 11-fold enrichment of the PM fraction relative to the crude membrane fraction was obtained and that the PM fraction was almost free of GA and ER contamination. The GA fraction was found to be enriched more than 5-folds with no significant cross-contamination by either the PM or ER as judged by the activity of glucan synthetase I. However, some contamination of ER in GA cannot be ruled out completely since relatively small activity of the dolichol monophosphate-GDP-mannose : mannosyl transferase was found in the GA fraction. The ER fraction was found to exhibit maximum activity of the marker enzymes mannosyl transferase and glucose-6-phosphatase and appeared to be virtually free of any PM contamination. Thus, by using differential centrifugation with sucrose gradients it was entirely possible to obtain sufficiently pure PM, GA and ER membrane fractions.

4.3 Subcellular distribution of APase in the various membrane fractions of peanut cotyledons

Table V shows the subcellular distribution of APase in germinating peanut cotyledons. The total crude membrane fraction (12,000xg - 105,000xg pellet), containing the PM, GA and ER membranes, was used as the starting material for the separation of various membrane fractions. Results show that a major portion (66.6%) of the total precipitable APase activity was associated with the PM fraction. The GA fraction contained 20.2% and the ER membrane fraction accounted for 13.2% of the remaining APase activity. In terms of specific activity also, the PM fraction showed the maximum activity (40.9 μM/min/mg) followed by GA

TABLE IV

DISTRIBUTION OF MARKER ENZYMES IN VARIOUS MEMBRANE FRACTIONS OF Arachis hypogaea COTYLEDONS

Fraction	Activity of marker enzymes (Radioactivity incorporated) Glucan synthetase I (cpm/mg)	Glucan synthetase II (cpm/mg)	Mannosyl transferase[a] (cpm/mg)	Glucose-6-phosphatase (µmol Pi/min/mg)
Crude membrane fraction (12,000 - 105,000xg pellet)	3,314	8,100	61,288 (21,000)	30.5[b]
Plasma membranes	1,230	88,246	3,080 (1,683)	13.36
Golgi apparatus	17,856	1625	18,661 (7208)	19.8
Smooth endoplasmic reticulum	1,972	3224	5,33,120 (38199)	39.4

[a] Values in parentheses were obtained without dolicholmonophosphate as exogenous lipid acceptor of mannose from GDP-[^{14}C] mannose as donor substrate.

[b] The activity observed was higher than the actual glucose-6-phosphatase activity as the enzyme preparation contains other non-specific phosphatases also.

TABLE V

SUBCELLULAR DISTRIBUTION OF ACID PHOSPHATASE IN PEANUT COTYLEDON CELLS

Various membrane fractions were isolated as described under Experimental Procedures. Percent yields were computed relative to the crude microsomal membrane fraction which was taken as 100%.

Fraction	Total protein (mg)	Total APase activity (μmol/min)	Specific activity (μmol/min/mg)	Yield (%)
Crude microsomal membrane fraction	428.1	7332	17.1	100
Plasma membranes	115.2	4722	40.9	66.6
Smooth endoplasmic reticulum	70.3	1432	20.3	20.2
Golgi apparatus	50.4	932	18.4	13.2

(20.3 µM/min/mg) and ER (18.4 µM/min/mg).

4.4 Isoenzymic pattern of APase in the plasma membranes of germinating peanut cotyledons

Fig. 3 shows the elution profiles of APase isoenzymes from the PM fraction of peanut cotyledons at various stages of germination (0-days, 2-days and 7-days) by CM-Sephadex column chromatography. Three isoenzymes of APase (APase I, APase II and APase III) eluted at 0.0 mM, 75 mM and 125 mM NaCl gradients, respectively, from the 0-days and 2-days germinated cotyledons, whereas, only two isoenzymes (APase I and APase III) eluted at 0.0 mM and 120 mM NaCl gradients from 7-days germinated cotyledons. The APase II isoenzyme appeared to be absent from the plasma membranes of 7-day old cotyledons.

Changes were also observed in the levels of APase isoenzymes present in the PM fraction with progressive germination period (Table VI). The level of APase I increased throughout the germination period but APase II activity disappeared completely within 7-days of germination. On the other hand, APase III following an initial decrease, exhibited a substantial increase in activity during the same germination period. Thus, it appears that the number and relative levels of activities of PM-APase isoenzymes of peanut cotyledons are dependent upon the physiological state of cotyledons.

4.5 Membrane-bound APase and 5'-nucleotidase activities in germinating peanut cotyledons

The variation in the activities of APase and 5'-nucleotidase associated with the microsomal membrane fraction (12,000xg - 105,000xg pellet) of peanut cotyledons during 14 days of germination is shown in Fig.4. Both enzymes exhibited maximum activity between 6th and 7th day of germination at 35°C. However,

FIGURE 3

Elution profiles of plasma membrane-bound acid phosphatase isoenzymes of peanut cotyledons from CM-Sephadex C-50 columns at various stages of germination. *The plasma membrane (PM) fractions from 0-days, 2-days and 7-days germinated peanut cotyledons were isolated and solubilized as described in the text. The solubilized PM fractions were dialyzed overnight against 100 volumes of 50 mM sodium acetate buffer (pH 5.0) and 4 ml (15 mg protein) of each of the dialyzed PM fraction loaded on to separate CM-Sephadex C-50 columns (1.5x20 cm) that were previously equilibrated with the same buffer. The columns were washed twice with equilibration buffer (twice the bed volume) to remove the unabsorbed proteins. The absorbed proteins were eluted by a linear gradient of 0-500 mM NaCl using a single mixing container with 50 ml of 50 mM sodium acetate buffer and a reservoir with 50 ml of the same buffer containing 500 mM NaCl. 1 ml fractions were collected at a flow rate of 0.2 ml/min and aliquots (0.2 ml) from every other fraction analyzed for the enzyme activity (●--●) and protein content (O--O).*

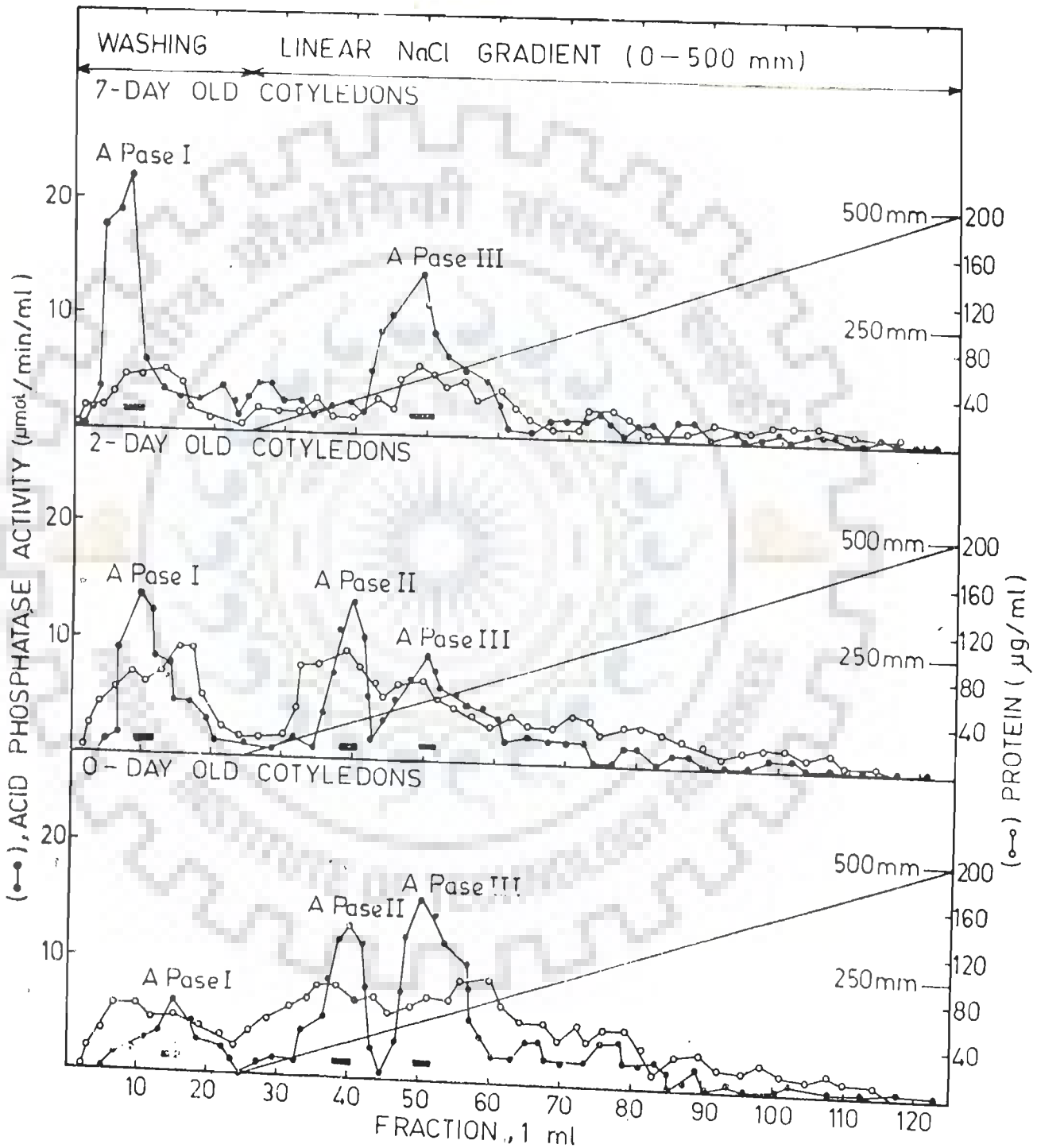

WASHING
LINEAR NaCl GRADIENT (0–500 mm)
7-DAY OLD COTYLEDONS
A Pase I
A Pase III
500mm
250mm
2-DAY OLD COTYLEDONS
A Pase I
A Pase II
A Pase III
0-DAY OLD COTYLEDONS
A Pase I
A Pase II
A Pase III
(—•), ACID PHOSPHATASE ACTIVITY (μmol/min/ml)
(—o) PROTEIN (μg/ml)
FRACTION, 1 ml

TABLE VI

CHANGES IN THE LEVELS OF ACID PHOSPHATASE ISOENZYMES WITH INCREASING GERMINATION PERIOD

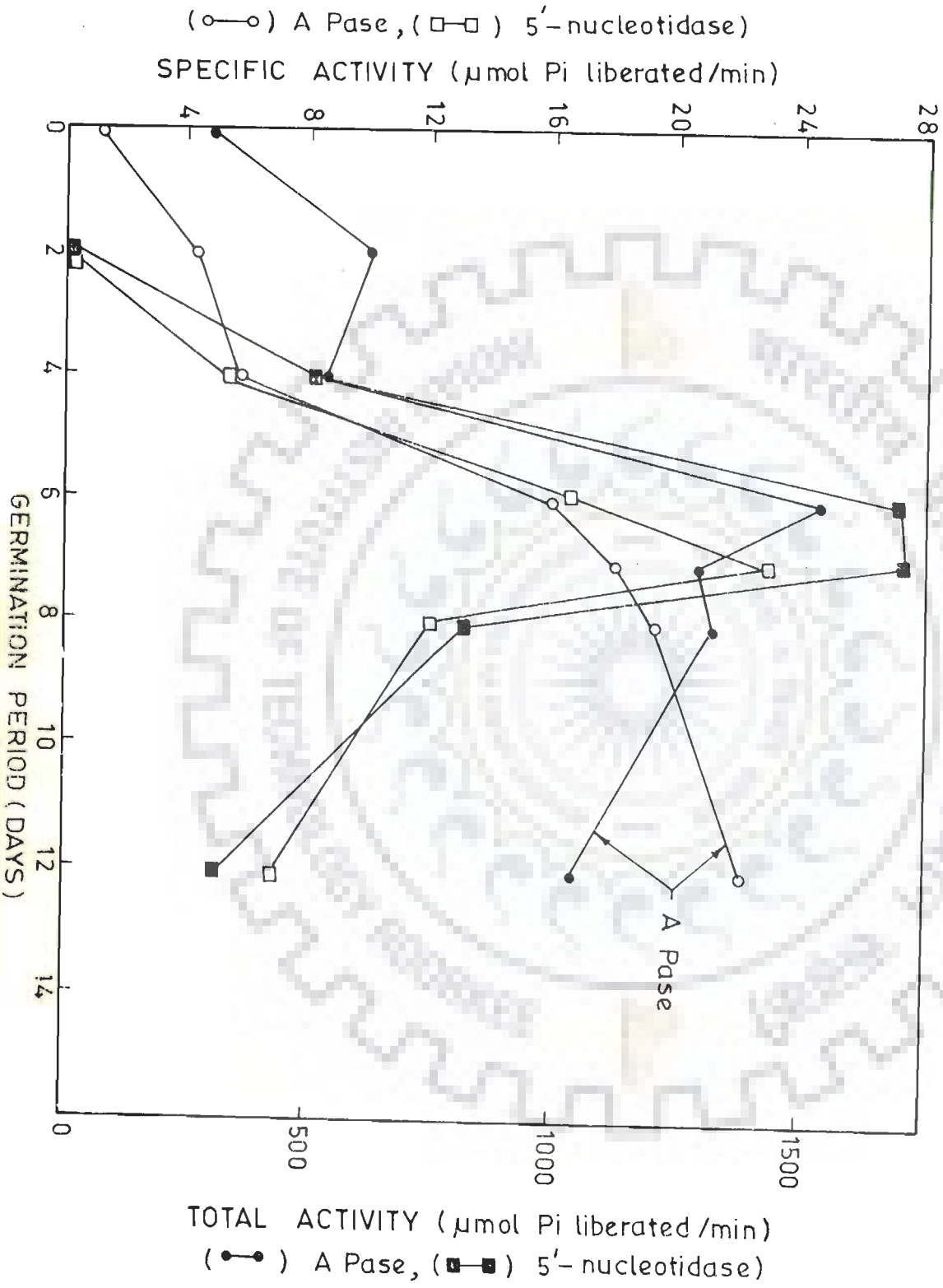
Plasma membrane fractions from 0-days, 2-days and 7-days old peanut cotyledons were prepared and solubilized as described in the text. The APase isoenzymes were separated by CM-Sephadex C-50 chromatography, pooled and their percent relative activities determined by assuming the total recoverable APase activity as 100%.

Period of germination	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)	Relative activity (%)
0-days				
APase I	1.30	148	113.8	27.0
Apase II	1.28	189	147.6	34.4
APase III	1.10	212	192.7	38.6
2-days				
Apase I	1.23	225	182.9	39.1
APase II	1.26	200	158.7	34.8
APase III	0.98	149	152.0	25.9
7-days				
Apase I	1.02	342	335.2	57.4
APase III	1.2	259	215.0	42.6

[a] Based on chromatographic behaviour on CM-Sephadex C-50 column

FIGURE 4

Effect of germination period on the levels of membrane-bound acid phosphatase and 5'-nucleotidase. *Peanut seeds were germinated in dark at 35°C and 100% humidity for the indicated time periods. 200 cotyledons (80-100 g, fresh wt.) were excised, washed and membrane fractions (12,000-105,000xg pellet) prepared as described under Experimental Procedures. Acid phosphatase (●--●) and 5'-nucleotidase(■-■) activities were assayed as described in the text.*


(o—o) A Pase, (□—□) 5′-nucleotidase)
SPECIFIC ACTIVITY (µmol Pi liberated/min)
0
4
8
12
16
20
24
28
GERMINATION PERIOD (DAYS)
0
2
4
6
8
10
12
14
A Pase
0
500
1000
1500
TOTAL ACTIVITY (µmol Pi liberated/min)
(•—•) A Pase, (■—■) 5′-nucleotidase)

the developmental patterns of the two enzymes were markedly different. For instance, 5'-nucleotidase emerged only after 48 h of imbibition, the period by which the APase had attained 40% of the maximal level. Similarly, while after 14 days of germination, the 5'-nucleotidase activity was insignificant, that of APase was substantially high. The specific activity was even higher. Thus, by using 2-days germinated peanut cotyledons, it was possible to prepare the desired membrane fractions devoid of 5'-nucleotidase activity for the purpose of isolation of APase.

4.6 Purification of PM-APases

The 2-days old peanut cotyledons were used as the starting material for isolating the PM-APase isoenzymes. The PM-APases were purified by following a relatively simple purification scheme involving only four main steps, namely, preparation of high specific activity PM fraction, solubilization of APases by a suitable detergent and two subsequent ion-exchange chromatography on CM-Sephadex C-50 and Sephadex G-150 resins, respectively.

Solubilization of PM-APases was effected by five different detergents to find out the detergent best suited for solubilizing the PM-APases. Results in Table VII show that of the five detergents (deoxycholate, taurocholate, triton X-100, nonidet P-40 and n-octylglucoside) used, n-octylglucoside was clearly the best one for the solubilization of PM-APases. Under the conditions employed, nearly two-third of the total enzyme activity was solubilized by octylglucoside and the specific activity increased about 6.7 folds. Triton X-100 and nonidet P-40 were only half as effective as octylglucoside. The ionic detergents, deoxycholate and taurocholate, were strongly inhibitory. Since the critical micellar concentration of n-octylglucoside is quite high (7.5 mg/ml), it was also possible to remove the detergent by dialysis before ion-exchange chromatography to avoid

TABLE VII

SOLUBILIZATION OF PLASMA MEMBRANE-BOUND ACID PHOSPHATASE WITH DIFFERENT DETERGENTS

For solubilization of the plasma membrane-bound acid phosphatases, the plasma membrane fraction (10.75 mg protein) was treated with 0.5% detergent at a protein-to-detergent ratio of 2:3 in presence of 20 mM $MgCl_2$ and 5 mM EDTA followed by centrifugation at 105,000xg for 60 min. The supernatant was assayed for APase activity. Total activity was expressed as µmol-pNP formed per min.

Fraction	Specific activity (µmol pNP/min/mg)	Activity solubilized (%)	Protein solubilized (%)
Plasma membrane	21.20	-	-
Detergent extract[a]			
Deoxycholate	4.45	4.8	8.4
Taurocholate	6.18	7.2	8.8
Triton X-100	29.92	28.4	9.5
Nonidet P-40	31.25	29.2	10.2
n-octylglucoside	60.60	68.9	10.3

[a] 105,000 x g supernatant

TABLE VIII

EFFECT OF Mg^{2+} AND EDTA ON THE SOLUBILIZATION OF APase FROM THE MEMBRANE FRACTION OF PEANUT COTYLEDONS

Membrane fraction (12,000 - 105,000xg pellet) was extracted with 0.5% n-octylglucoside without and with $MgCl_2$ and EDTA as described under Experimental Procedures. The homogenate was centrifuged at 105,000xg for 60 min and the supernatant fraction used as solubilized enzyme. The APase activity was assayed in a routine manner. Values expressed below are the average of three separate experiments.

Conditions of solubilization EDTA (mM) + $MgCl_2$ (mM)		Protein solubilized (%)	APase activity solubilized (%)	Specific activity of APase (µmol pNP/min/mg)
0.0	0.0	24.2 ± 1.0	15.1 ± 1.0	8.5 ± 2.0
0.0	20.0	12.8 ± 1.0	14.4 ± 2.0	9.7 ± 1.0
5.0	0.0	11.2 ± 2.0	17.8 ± 1.5	12.4 ± 1.0
5.0	5.0	9.3 ± 1.5	19.2 ± 1.0	13.2 ± 2.0
5.0	10.0	10.0 ± 1.5	28.4 ± 0.5	11.2 ± 1.5
5.0	20.0	10.4 ± 0.5	35.4 ± 0.5	18.0 ± 1.5
5.0	25.0	10.2 ± 1.0	29.2 ± 1	15.4 ± 3.0

interference in the purification procedure.

Mg^{2+} and EDTA were found to be essential for selective solubilization of the enzyme from the PM (Table VIII). Thus, solubilization of PM-APases was done with the nonionic detergent n-octylglucoside in the presence of 20 mM $MgCl_2$ and 5 mM EDTA at a protein-to-detergent ratio of 2:3 as described under Experimental Procedures. The solubilized APase was dialyzed 16 h against 50 mM acetate buffer (pH 5.0) and loaded (20 mg protein) on CM-Sephadex C-50 column.

Fig.5A shows the elution profile of PM-APases from CM-Sephadex C-50 column. Three isoenzymes of APase were eluted at 0.0 mM, 75 mM and 125 mM NaCl gradients and were designated as APase I, APase II and APase III, respectively. Of the three isoenzymes, APase I showed maximum APase activity. Each isoenzyme was again subjected to gel filtration by Sephadex G-150 chromatography. The gel filtrations were carried out at pH 5.0. All the three isoenzymes emerged as single peaks from Sephadex G-150 columns (Fig.5B). Maximum specific activity was exhibited by APase I (208.1 µmol/min/mg) followed by APase II (181.6 µmol/min/mg) and APase III (170.6 µmol/min/mg). Their respective purification folds were 12.1, 10.6 and 9.9. The entire purification scheme of PM-APases has been summarized in Table IX. Although the yield of APase I, APase II and APase III (1.5%, 1.4% and 1.0%, respectively) was lowered considerably during the various steps of purification, the purification scheme described here resulted in homogeneous preparations of PM-APase isoenzymes as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

4.7 Homogeneity of purified PM-APases

The purified PM-APases were found to be homogeneous as indicated by the presence of single protein bands when subjected to SDS-PAGE in the presence of β-mercaptoethanol. The apparent molecular weights of APase I, APase II and APase III were estimated to be 79 Kda, 76 Kda and 66 Kda, respectively.

FIGURE 5A

Elution profile of plasma membrane-bound acid phosphatase isoenzymes from CM-Sephadex C-50 column. *The plasma membrane fraction from 2-days germinated peanut cotyledons was solubilized by the detergent n-octylglucoside as described under Experimental Procedures. The solubilized PM fraction was dialyzed 16 h against 100 volumes of 50 mM sodium acetate buffer (pH 5.0) and 5 ml (20 mg protein) loaded on a CM-Sephadex C-50 column (1.5x20 cm) which was preequilibrated with the same buffer. The conditions for elution were the same as described in the legend of FIGURE 3. Aliquots (0.2 ml) from every alternate fraction were analyzed for acid phosphatase activity (O--O) and protein content (●--●). Top few fractions of each peak as indicated by the bar (—) were pooled,dialyzed and concentrated by ultrafiltration and used for further purification by gel filtration.*

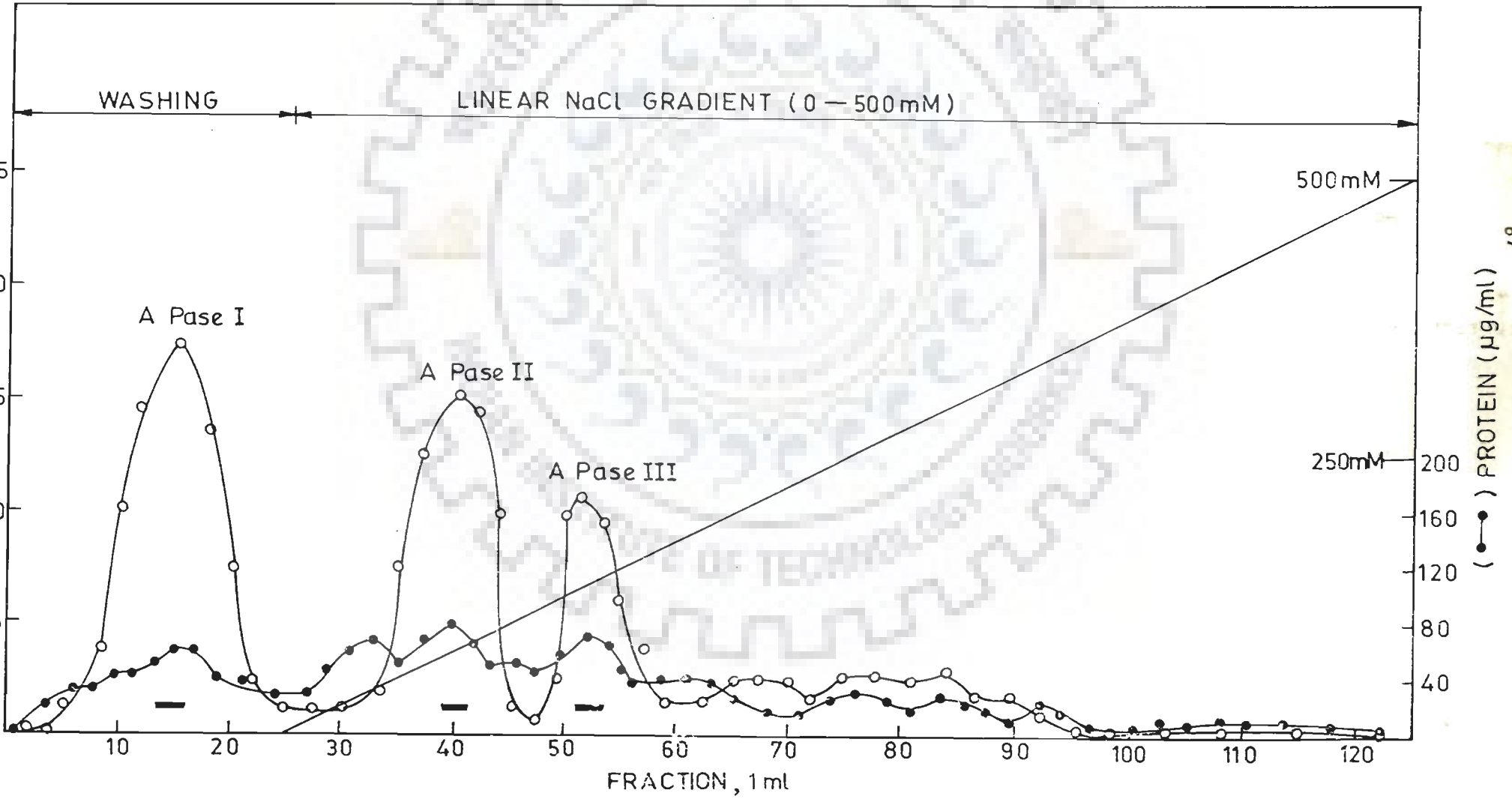

WASHING
LINEAR NaCl GRADIENT (0—500mM)
500mM
250mM
A Pase I
A Pase II
A Pase III
( ) PROTEIN (µg/ml)
200
160
120
80
40
10
20
30
40
50
60
70
80
90
100
110
120
FRACTION, 1ml

FIGURE 5B

Elution profiles of plasma membrane-bound acid phosphatase isoenzymes from Sephadex G-150 columns equilibrated at pH 5.0. *The enzyme fractions (APase I, APase II and APase III) obtained by CM-Sephadex C-50 chromatography were dialyzed overnight against 100 volumes of 50 mM sodium acetate buffer (pH 5.0), concentrated by ultrafiltration and loaded on separate Sephadex G-150 columns (0.9x25 cm) that were previously equilibrated with the same buffer. 1 ml fractions were collected at a flow rate of 1 ml/15 min and aliquots (0.2 ml) from every other fraction were analyzed for acid phosphatase activity (O--O) and protein content (●--●). The fractions indicated by the bar (▬) were pooled and used for further studies.*

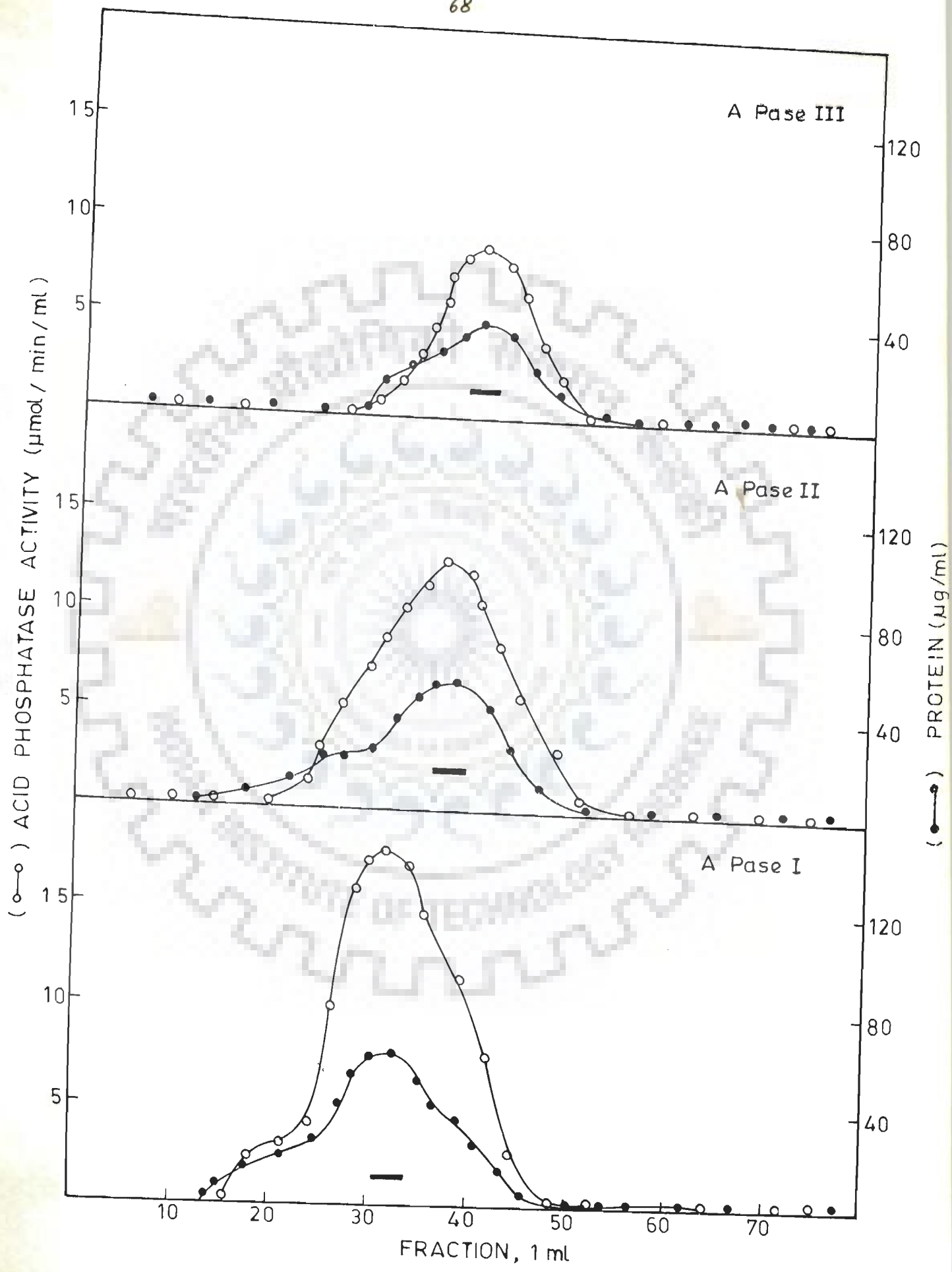

A Pase III
A Pase II
A Pase I
15
10
5
120
80
40
10
20
30
40
50
60
70
FRACTION, 1 ml
(—o—) ACID PHOSPHATASE ACTIVITY (µmol / min / ml)
(—•—) PROTEIN (µg/ml)

TABLE IX

PURIFICATION OF ACID PHOSPHATASES FROM THE PLASMA MEMBRANES OF PEANUT COTYLEDONS

Plasma membrane fraction was obtained from the crude particulate fraction (12,000 - 105,000xg pellet) from 2-days old peanut cotyledons as described in Experimental Procedures. The plasma membranes (115.2 mg protein) were extracted with 0.5% n-octylglucoside in presence of 20 mM $MgCl_2$ and 5 mM EDTA and centrifuged at 105,000xg for 60 min. The supernatant fraction was used as the solubilized form of enzyme. CM-Sephadex peaks I,II and III represent the pool of top six fractions while Sephadex G-150 peaks represent the pool of top three fractions of each peak. The purification and specific activity data were computed relative to the crude membrane fraction.

Fraction	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)	Purification (fold)	Yield (%)
Crude microsomal membrane fraction	428.1	7332	17.1	-	100
Plasma membrane fraction	115.2	4722	40.9	2.4	64.4
Octylglucoside extract	20.4	2314	113.4	6.6	31.5
CM-Sephadex C-50					
Peak I(APase I)	0.82	149.7	182.5	10.7	2.0
Peak II(APase II)	0.84	133.2	158.5	9.3	1.8
Peak III(APase III)	0.70	106.4	152.0	8.9	1.4
Sephadex G-150					
APase I	0.54	112.4	208.1	12.1	1.5
APase II	0.60	108.9	181.6	10.6	1.4
APase III	0.47	80.2	170.6	9.9	1.0

The SDS-PAGE patterns of the purified PM-APases are shown in Fig.6. Electrophoresis in native gels (in the absence of SDS and β-mercaptoethanol) also resulted in the appearance of single protein bands. Thus, the PM-APases appear to have only a single polypeptide chain each.

4.8 Formation of high molecular weight forms *in vitro*

When APase I, APase II and APase III were dialyzed for 48 h against 50 mM Tris-HCl buffer (pH 7.2) and then subjected to PAGE without SDS and β-mercaptoethanol, the isoenzymes gave two protein bands each (Fig 7B). When the pH of the same samples was adjusted back to pH 5.0 by dialyzing against 50 mM sodium acetate buffer (pH 5.0) and subjected to PAGE as before, again single bands were obtained (Fig. 7C). However, SDS-PAGE in all cases yielded single protein bands only (Fig. 8A, 8B, 8C). These results indicate that at pH 5.0, the isoenzymes exist in monomeric forms containing single polypeptide chains, but at pH 7.2 conversion of monomeric forms to higher molecular weight forms occurs, with subsequent dissociation into monomers on lowering the pH to 5.0 from pH 7.2. The higher molecular weight forms of APases at pH 7.2 were found to be dimers. That each dimer contained two polypeptide chains of identical molecular weight was indicated by the fact that SDS-PAGE performed under completely dissociating conditions yielded single protein bands (Fig. 8B) but two bands in each case on native PAGE (Fig. 7B). The molecular weight of the dimeric forms of APase I, APase II and APase III were found to be 159 Kda, 150 Kda and 130 Kda, respectively.

The formation of high molecular weight forms from monomeric forms *in vitro* was also confirmed by actually separating the two forms of each isoenzyme on Sephadex G-150 columns that were maintained at pH 7.2. After the elution of the three APase isoenzymes from CM-Sephadex C-50 column during purification

FIGURE 6

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified acid phosphatase isoenzymes. *SDS-PAGE of purified PM-APase isoenzymes was performed by the method of Weber and Osborn using an SDS-Phosphate buffer system as described under Experimental Procedures. SDS-PAGE was carried on 10% gels in cylindrical tubes (0.5x8.0 cm) at a constant current of 8 mA/tube at room temperature. Samples and amounts loaded were : APase I (40 µg), APase II (40 µg), APase III (40 µg) and standard proteins (5 µg each). The molecular weight standards were : Myosin, M_r = 200 Kda; β-galactosidase, M_r = 116 Kda; phosphorylase b, M_r = 94 Kda; bovine serum albumin, M_r = 68 Kda; ovalbumin, M_r = 43 Kda; carbonic anhydrase, M_r = 30 Kda; soybean trypsin inhibitor, M_r = 21 Kda and lysozyme, M_r = 14.3 Kda. The direction of electrophoresis was from top to bottom. The molecular weight of the purified isoenzymes were computed from the calibration plot of $\log_{10} M_r$ versus relative mobility of standard proteins. Frac.I (APase I) corresponded to molecular weight of 79 Kda, Frac. II (APase II) corresponded to molecular weight of 76 Kda and Frac. III (Apase III) corresponded to molecular weight of 66 Kda.*

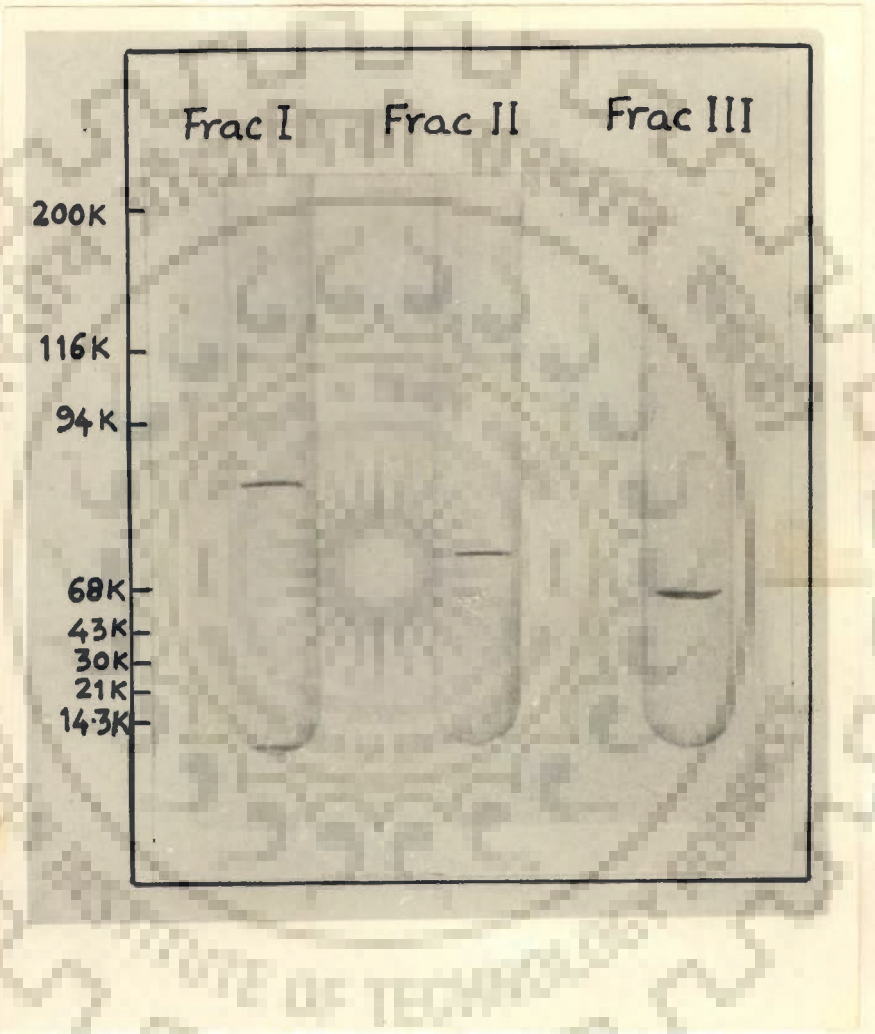

Frac I
Frac II
Frac III
200K
116K
94K
68K
43K
30K
21K
14·3K

FIGURE 7

Polyacrylamide gel electrophoresis (PAGE) of purified acid phosphatase isoenzymes kept at different pH. *Native PAGE of purified APase isoenzymes were performed similar to SDS-PAGE but without the presence of SDS and β-mercaptoethanol. Conditions for electrophoresis, staining and destaining of gels etc. were also identical to those of SDS-PAGE. Samples and amounts loaded were : APase I (35 µg), APase II (35 µg) and APase III (35 µg).*

Group 7A : Purified APase I (Frac.I), APase II (Frac.II) and APase III (Frac. III) were dialyzed 16 h against 50 mM sodium acetate buffer (pH 5.0) and suitable aliquots subjected to native PAGE.

Group 7B : The above three fractions of Group 7A were further dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.2) and again suitable aliquots subjected to native PAGE.

Group 7C : The above three fractions of Group 7B were dialyzed back for 16 h against 50 mM sodium acetate buffer (pH 5.0) and subjected to native PAGE.

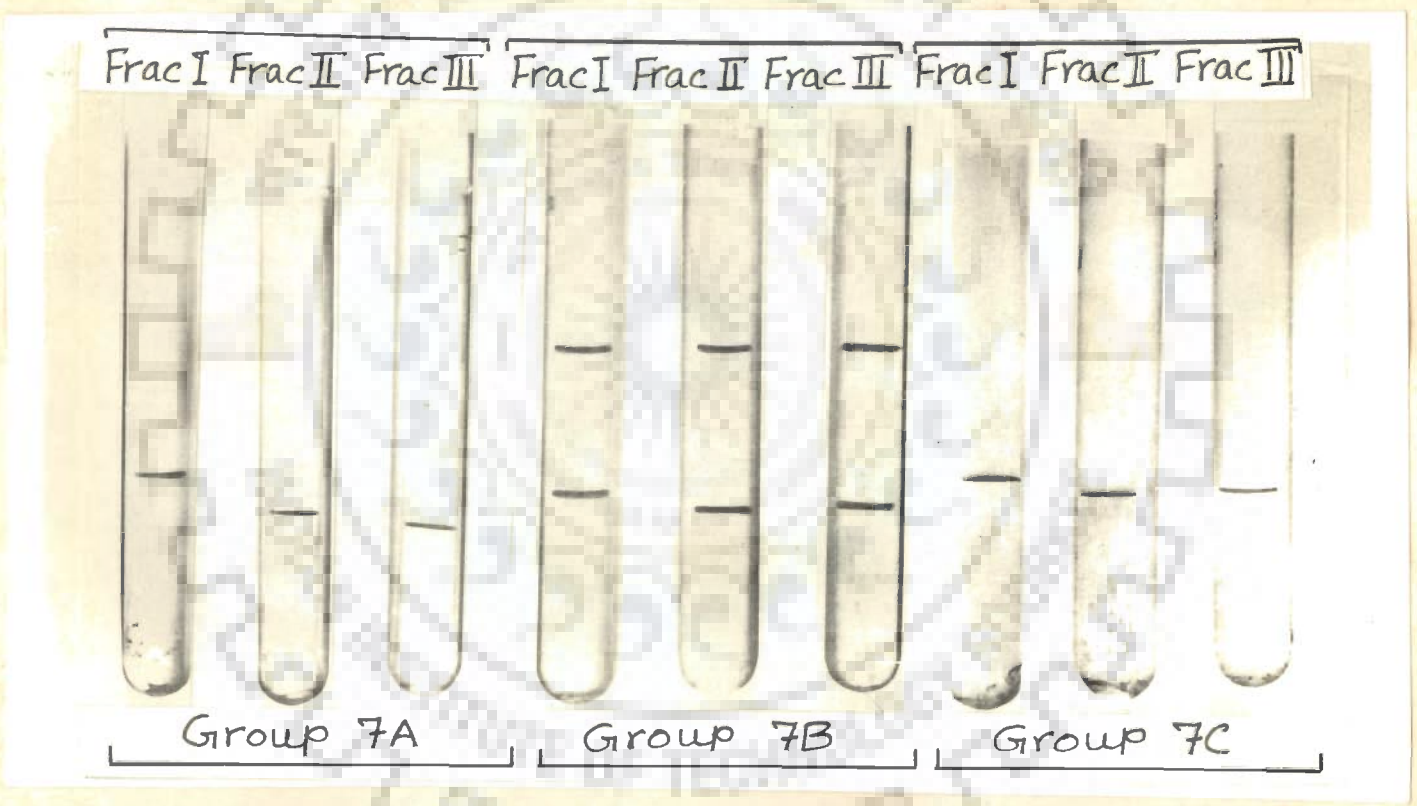

Frac I Frac II Frac III
Frac I Frac II Frac III
Frac I Frac II Frac III
Group 7A
Group 7B
Group 7C

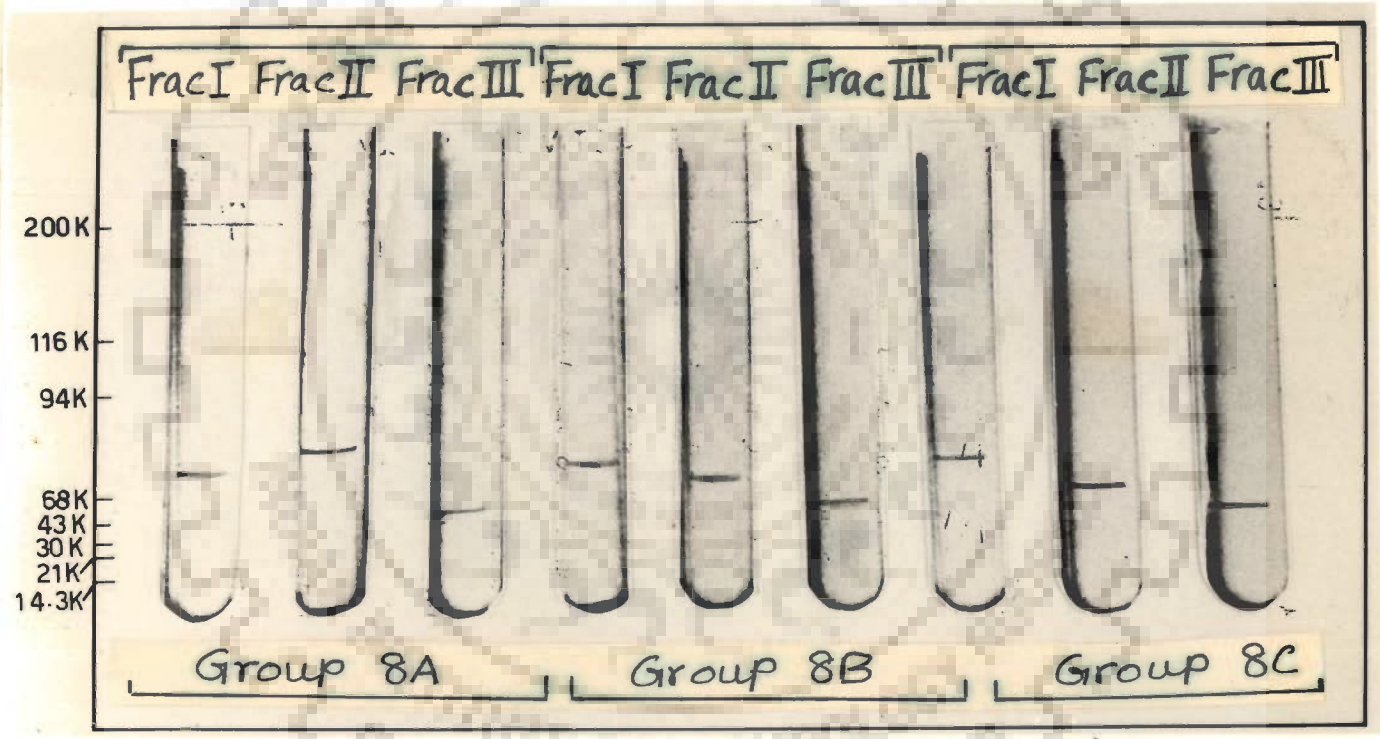
FIGURE 8

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of purified acid phosphatase isoenzymes kept at different pH. *Conditions for SDS-PAGE were exactly those as described in the legend of FIGURE 6. Samples and amounts loaded were : APase I (40 µg), APase II (40 µg) and APase III (40 µg). The direction of electrophoresis was from top to bottom.*

Group 8A : Purified APase I (Frac. I), APase II (Frac. II) and APase III (Frac. III) were dialyzed 16 h against 50 mM sodium acetate buffer (pH 5.0) and suitable aliquots subjected to SDS-PAGE.

Group 8B : The above three fractions of Group 8A were further dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.2) and again suitable aliquots subjected to SDS-PAGE.

Group 8C : The above three fractions of Group 8B were dialyzed back for 16 h against 50 mM sodium acetate buffer (pH 5.0) was subjected to SDS-PAGE as before.


Frac I Frac II Frac III Frac I Frac II Frac III Frac I Frac II Frac III
200K
116K
94K
68K
43K
30K
21K
14.3K
Group 8A
Group 8B
Group 8C

procedure, these were dialyzed 48 h against Tris-HCl buffer (pH 7.2) and chromatographed on Sephadex G-150 columns that were also equilibrated with the same buffer (pH 7.2). The elution profiles of APase I, APase II and Apase III from Sephadex G-150 columns at pH 7.2 are shown in Fig 9. Results clearly indicate that during dialysis against pH 7.2, the monomeric forms were associated into higher molecular weight forms to a large extent and these two molecular forms could be separated by Sephadex G-150 columns maintained at pH 7.2. These higher molecular weight forms of APase I, APase II and APase III (peaks IA, IIA and IIIA of Fig 9) were again found to be dimers as before with respective molecular weights of 162 Kda, 148 Kda and 125 Kda. When these dimeric forms were again dialyzed against pH 5.0 and subjected to gel filtration on Sephadex G-150 at pH 5.0, the elution pattern of these APases reverted back to that shown in Fig.5 and their monomeric character confirmed by SDS-PAGE and PAGE patterns. Thus, in vitro association and dissociation of PM-APases is a pH-dependent phenomenon. To avoid problems related to pH-dependent association exhibited by the PM-APase isoenzymes, the purified isoenzymes were stored at pH 5.0 to ensure their monomeric nature and unless mentioned otherwise, their characterization and other experiments involving the purified APase isoenzymes were carried out at pH 5.0.

4.9 Properties of purified PM-APases

4.9.1 Optimum pH and pH stability

The purified PM-APases gave identical pH-activity profiles exhibiting a single pH optimum at pH 5.0 (Fig 10). Below and above pH 5.0 the APase activity was found to decline sharply in all the three cases. The PM-APases also exhibited maximum stability at pH 5.0 (Fig 11).

4.9.2 Kinetic properties

The K_m and V_{max} values of APase I, APase II and APase III for the hydrolysis of p-nitrophenyl phosphate as determined by Lineweaver-Burk plots

FIGURE 9

Elution profiles of plasma membrane-bound acid phosphatase isoenzymes from Sephadex G-150 columns equilibrated at pH 7.2. *The enzyme fractions (APase I, APase II and APase III) obtained after CM- Sephadex C-50 chromatography were dialyzed overnight against 100 volumes of 10 mM Tris-HCl buffer (pH 7.2), concentrated by ultrafiltration and loaded on separate Sephadex G-150 columns (0.9 x 25 cm) that were preequilibrated with 10 mM Tris-HCl (pH 7.2). 1 ml fractions were collected at a flow rate of 1 ml/15 min and aliquots (0.2 ml) from every other fraction analyzed for acid phosphatase activity (O--O) and protein content (●--●). This gel filtration step was seen to resolve APase I, APase II and APase III into two distinct peaks each that were designated as IA and IB ; IIA and IIB; IIIA and IIIB respectively.*

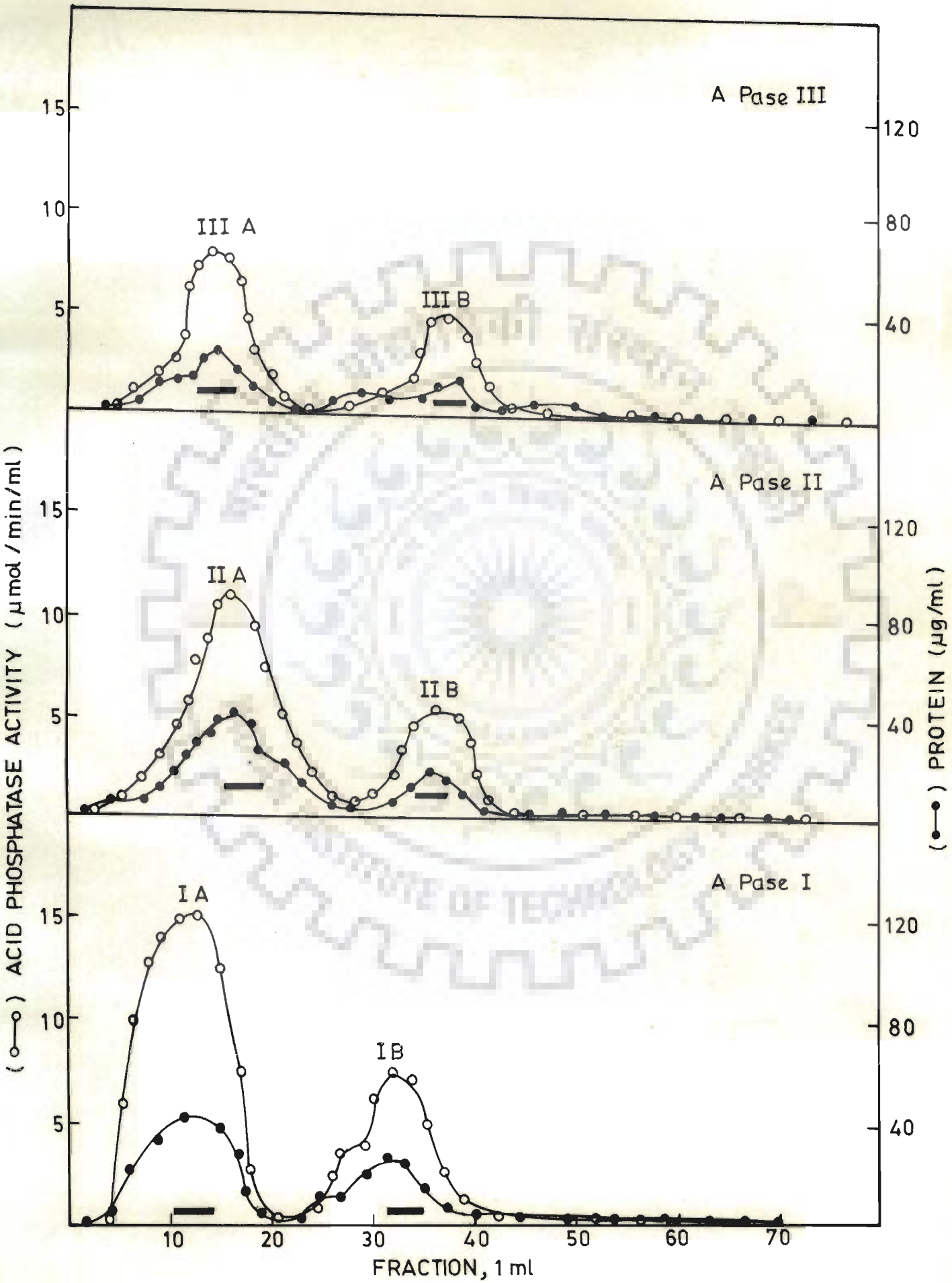

A Pase III
III A
III B
A Pase II
II A
II B
A Pase I
I A
I B
(o—o) ACID PHOSPHATASE ACTIVITY (μmol/min/ml)
(•—•) PROTEIN (μg/ml)
FRACTION, 1 ml
15
10
5
120
80
40
10
20
30
40
50
60
70

FIGURE 10

pH-activity profiles of purified acid phosphatase isoenzymes. *The enzyme activities of APase I (■—■), APase II (●---●) and APase III (O---O) were measured at different pH in standard assay systems. A fixed amount of enzyme protein (50 µg) was incubated with 2 mM p-nitro phenyl phosphate at 30°C for 15 min in suitable buffers of varying pH. The buffers used were 50 mM sodium acetate buffer (pH 3.5 - 5.5) and 50 mM Tris-HCl buffer (pH 6.0 - 9.5). Control assays at each pH were run simultaneously. Enzyme activities were represented as percentage of maximum activity.*

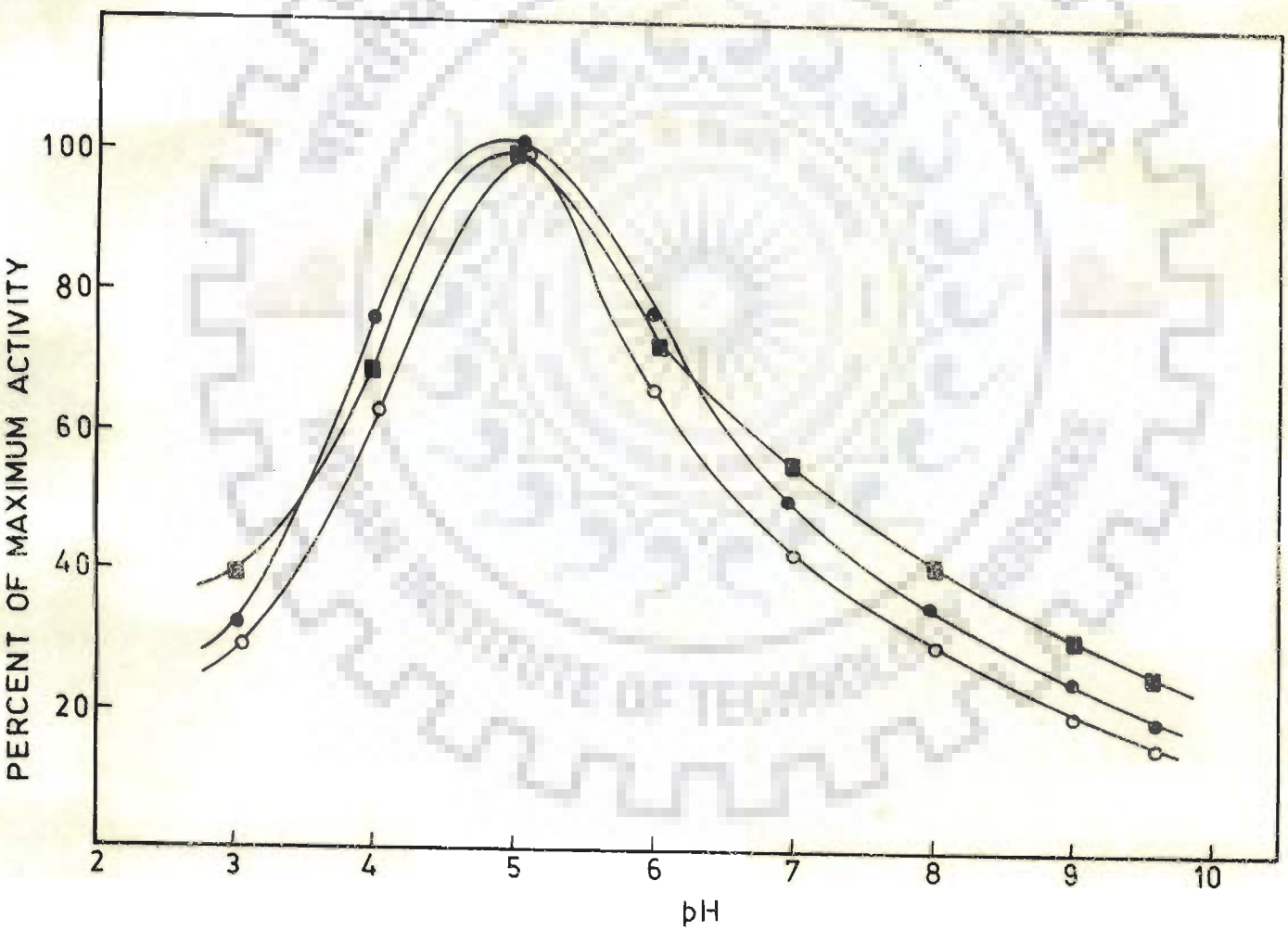
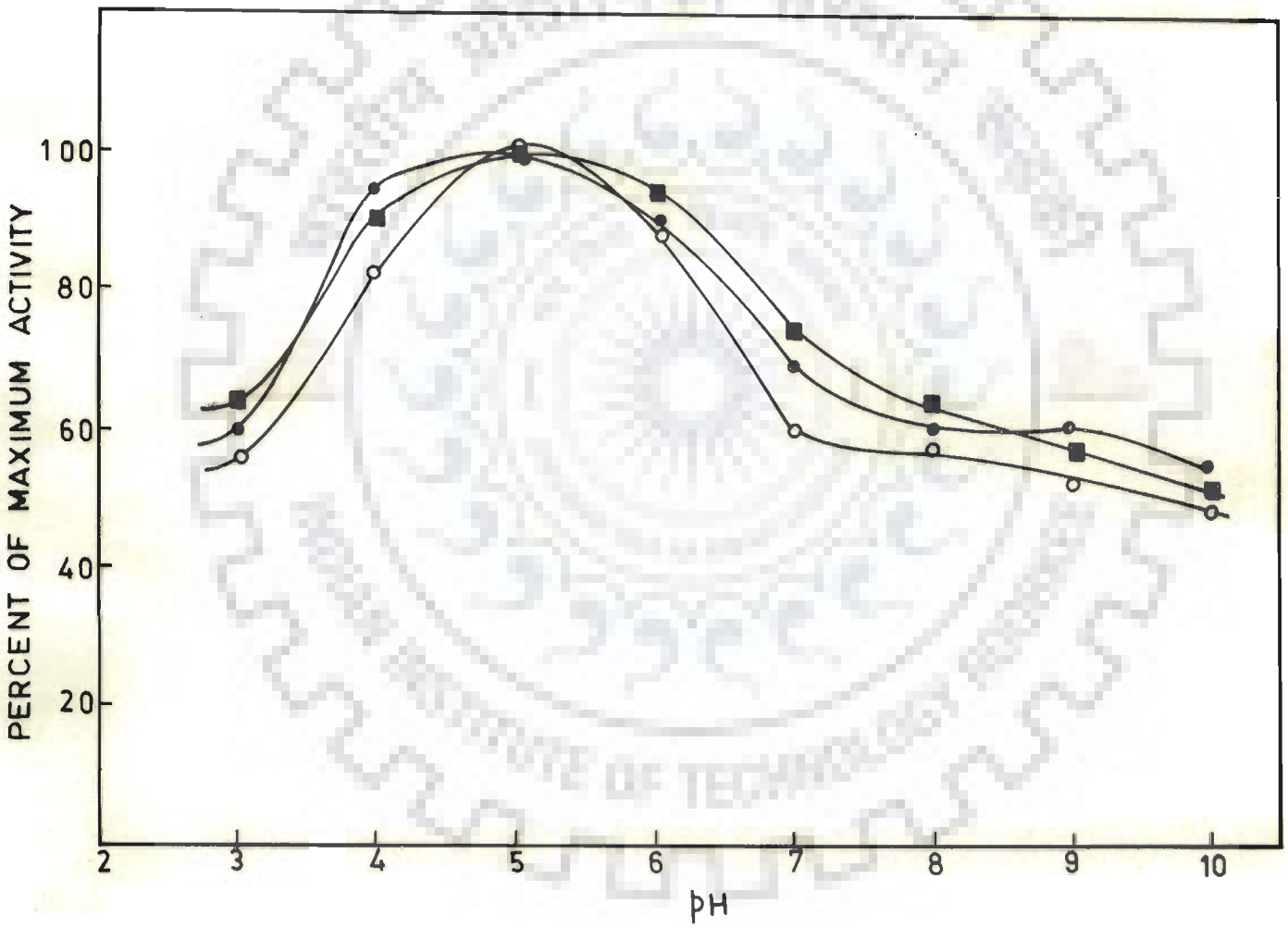

PERCENT OF MAXIMUM ACTIVITY
100
80
60
40
20
2
3
4
5
6
7
8
9
10
pH

FIGURE 11

pH-stability profiles of purified acid phosphatase isoenzymes. *Purified APase I (■–■), APase II (●--●) and APase III (O--O) were preincubated with suitable buffers of varying pH at 4°C for 3 h after which the enzyme activities were assayed in a normal manner. The buffers used were 50 mM sodium acetate (pH 3.5 - 5.5) and 50 mM Tris-HCl (pH 6.0 - 9.5). Control assays at each pH were run simultaneously. Enzyme activities were represented as percentage of maximum activity.*


PERCENT OF MAXIMUM ACTIVITY
100
80
60
40
20
2
3
4
5
6
7
8
9
10
pH

were 0.43 mM, 0.50 mM, 0.48 mM and $2.1x10^2$ µM/min/mg, $2.8x10^2$ µM/min/mg and $2.4x10^2$ µM/min/mg respectively (Fig 12). Orthophosphate (PO_4^{3-}) inhibited APase I (K_i = 1.42 mM) and APase II (K_i = 0.83 mM) competitively, but APase III was inhibited noncompetitively (K_i = 0.48 mM) (Fig 13).

4.9.3 Substrate specificity

The substrate specificities of the purified APase isoenzymes were tested at pH 5.0 and pH 7.2 using GMP, UMP, CMP, AMP, UDP, ADP, GDP, GTP, ATP, CTP, glucose-1-phosphate (glu-1-P), glucose-6-phosphate (glu-6-P), glyceraldehyde-3-phosphate (gly-3-P) and para nitro phenyl phosphate (pNPP) as substrates. The data are compared in Table X. All the isoenzymes showed broad substrate specificity which was pH-dependent with degree of specificity for each isoenzyme being significantly different. For instance, while at pH 5.0 APase I, APase II and APase III showed no activity towards GMP, AMP, UMP and glu-6-P, all these substrates, exoept UMP, were substantially hydrolyzed at pH 7.2. In addition the results showed that at pH 7.2, the APase isoenzymes exhibit greater substrate specificity towards nucleotide phosphates and sugar phosphates than towards pNPP. However at pH 5.0, the isoenzymes show maximal activity towards pNPP.

4.9.4 Effect of various cations and anions

Table XI shows the effect of some common cations and anions on the APase isoenzymes. Ni^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mo^{2+}, F^- and PO_4^{3-} inhibited APase I, APase II and APase III, although the degree of inhibition was different for different APase isoenzymes. The effect of Mg^{2+}, Mn^{2+}, Mg^{2+}, tartarate and citrate ions on various isoenzymes was highly differential. For instance, Mg^{2+} strongly inhibited APase III, but had no effect on APase I and APase II. Likewise, APase I was strongly inhibited by Mn^{2+} and Hg^{2+} but APase II and APase III were not inhibited. Interestingly, tartarate and citrate ions were found to activate

FIGURE 12

Lineweaver-Burk plots of purified acid phosphatase isoenzymes using p-nitro phenyl phosphate as substrate. *Enzyme assays were carried out in a normal manner with varying amounts of the substrate p-nitro phenyl phosphate (0.25 mM - 2 mM) and fixed amount of enzyme protein (50 µg). K_m and V_{max} values of APase I (■—■), APase II (●--●) and APase III (O--O) were computed from the intercepts on X-axes and Y-axes, respectively.*

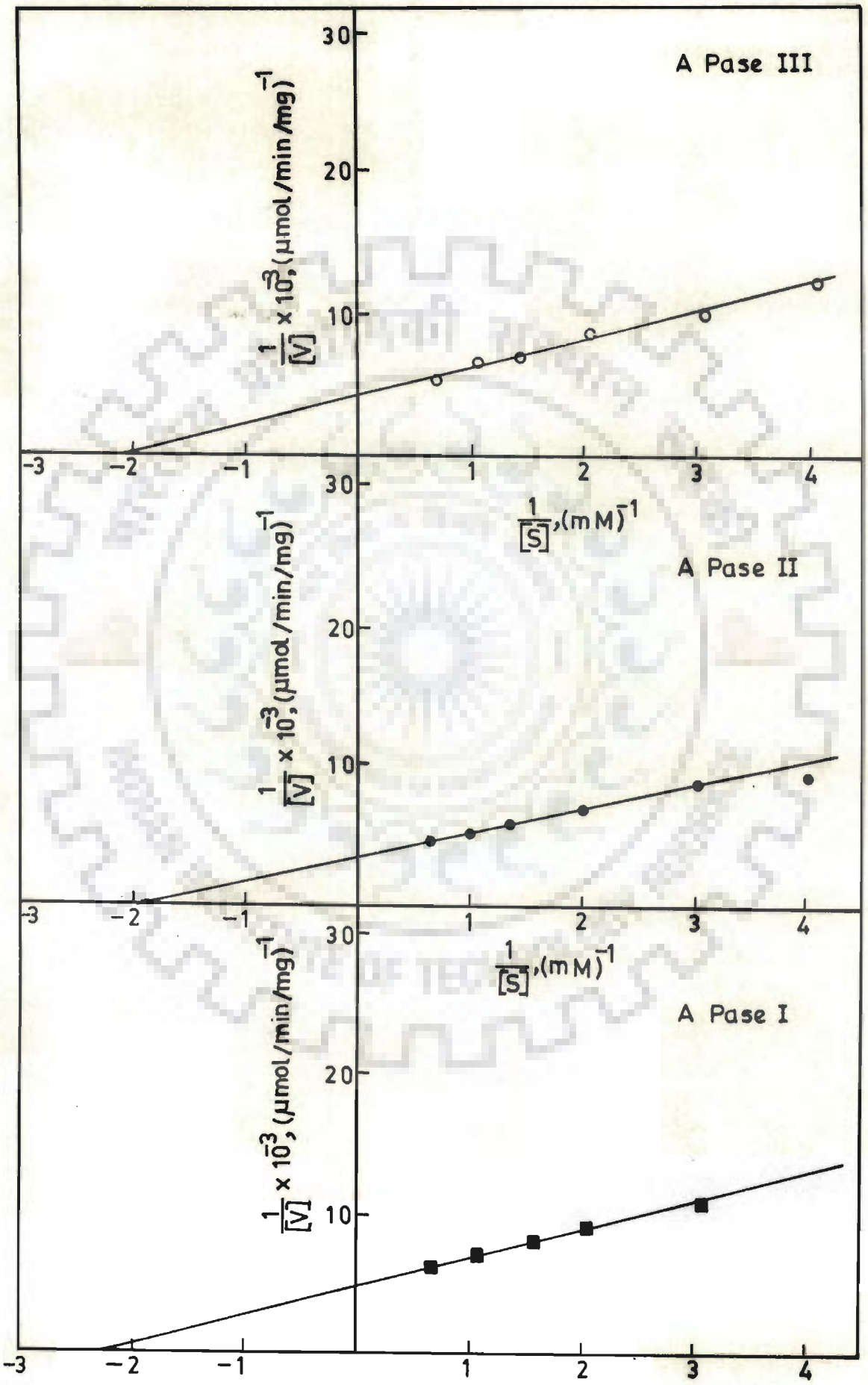

A Pase III
A Pase II
A Pase I
$\frac{1}{[V]} \times 10^{-3}$, (µmol/min/mg)$^{-1}$
$\frac{1}{[S]}$, (mM)$^{-1}$
30
20
10
-3
-2
-1
1
2
3
4

FIGURE 13

Lineweaver-Burk plots of purified acid phosphatase isoenzymes showing inhibition by orthophosphate. *Enzyme assays were carried out in a normal manner with varying amounts of the substrate p-nitro phenyl phosphate (0.25 mM - 2 mM) in the presence (●--●) and absence (O--O) of a fixed concentration (0.5 mM) of the inhibitor sodium dihydrogen orthophosphate. Values of inhibition constant (K_i) of the APase isoenzymes were computed from the intercepts on X-axes of plots obtained in the presence (O--O) of the inhibitor.*

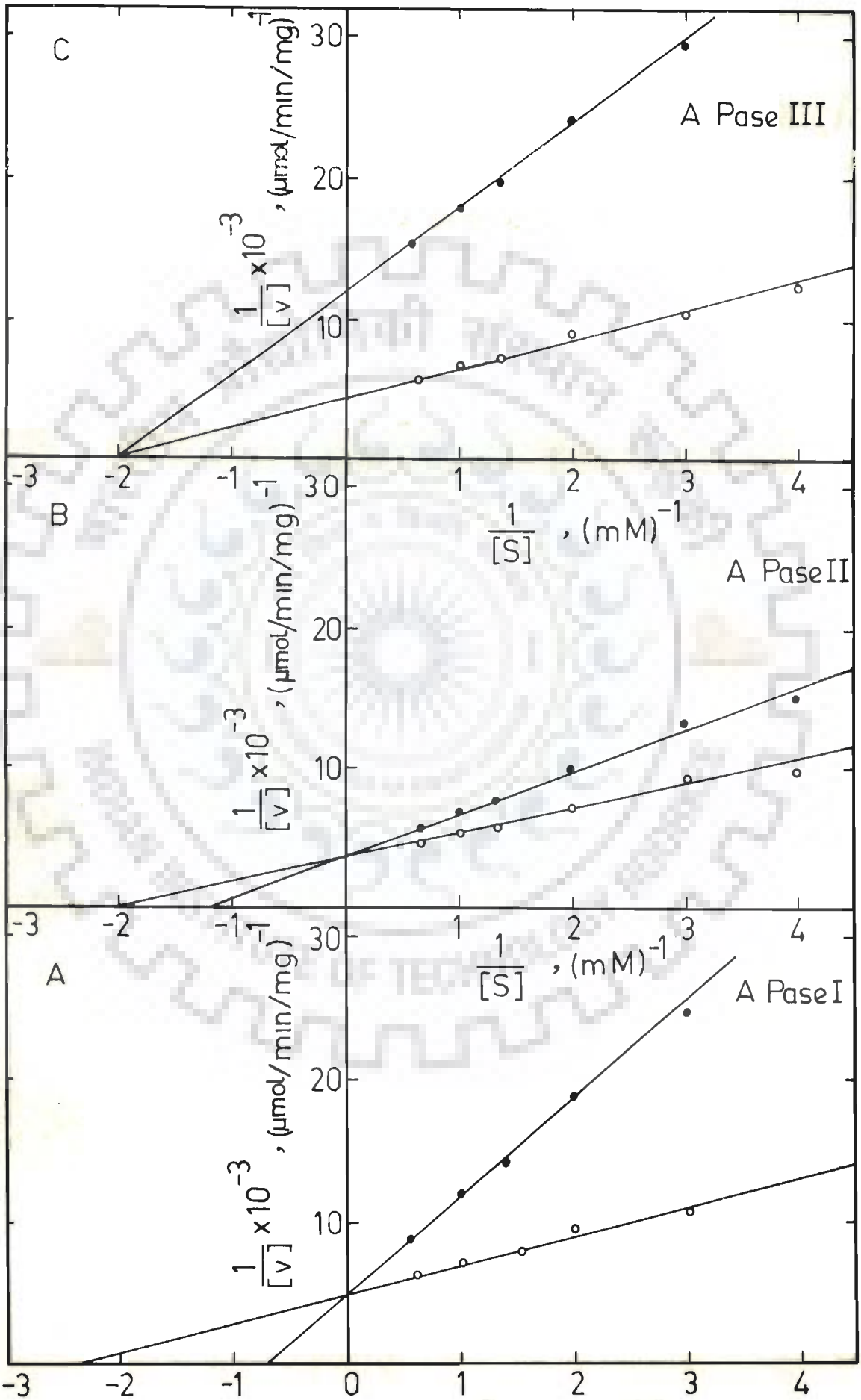

C
A Pase III
B
A PaseII
A
A PaseI
$\frac{1}{[v]} \times 10^{-3}$, (µmol/min/mg)$^{-1}$
$\frac{1}{[S]}$, (mM)$^{-1}$
-3
-2
-1
0
1
2
3
4
10
20
30

TABLE X

SUBSTRATE SPECIFICITY OF THE PURIFIED APase ISOENZYMES

The enzyme assays were performed at pH 5.0 and pH 7.2 using 1 mM of the indicated substrates as described under Experimental Procedures.

	pH 5.0			pH 7.2		
	Specific Activity (μM/min/mg)			Specific Activity (μM/min/mg)		
Substrate	APase I	APase II	APase III	APase I	APase II	APase III
ATP	40.6	100.4	119.1	63.3	79.6	157.2
GTP	32.5	85.6	115.5	102.9	226.7	281.7
CTP	36.5	5.9	86.6	102.9	110.3	190.0
ADP	8.1	70.9	97.5	79.1	110.3	216.2
UDP	32.5	47.2	46.9	79.1	110.3	176.8
GDP	32.5	79.7	79.4	39.5	61.2	104.8
AMP	0.0	0.0	0.0	63.3	12.2	32.7
GMP	0.0	0.0	0.0	63.3	12.2	32.7
CMP	52.8	5.9	0.0	63.3	12.2	32.7
UMP	0.0	0.0	0.0	39.5	0.0	13.1
Gly-3-P	40.6	70.9	133.6	102.9	134.8	301.3
Glu-1-P	32.5	5.9	0.0	419.5	12.2	0.0
Glu-6-P	0.0	0.0	0.0	63.3	12.2	19.6
pNPP	89.3	141.8	155.2	102.9	147.0	216.2

TABLE XI

EFFECT OF SOME CATIONS AND ANIONS ON THE PURIFIED APase ISOENZYMES

The enzyme assays were performed at pH 5.0 using 10 mM concentration of the indicated cations and anions. Control reaction mixtures did not contain the respective ion. Percent activities of APase were calculated by considering the activity of the control as 100%.

Cations and anions (10 mM)	Residual enzyme activity, % of control		
	APase I	Apase II	APase III
Control	100	100	100
Ni^{2+}	51.6	78.5	42.8
Mg^{2+}	100	100	42.8
Mn^{2+}	24.1	100	95.3
Ca^{2+}	34.5	75.6	95.3
Zn^{2+}	34.5	75.6	4.6
Hg^{2+}	24.1	100	100
PO_4^{3-}	34.5	25.6	11.8
F^-	17.1	52.8	30.8
Cu^{2+}	0.0	57.1	0.0
Mo^{2+}	0.0	0.0	0.0
Tartarate	100	132.7	152.5
Citrate	96.6	125.6	143.0

APase II and APase III but not APase I. Thus, it seems that the APase isoenzymes (APase II and APase III), which were not inhibited by Mn^{2+} or Hg^{2+} were activated by tartarate and citrate ions, and in contrast APase I, which is sensitive to Mn^{2+} and Hg^{2+}, was not activated. Whether there is some correlation between the inhibitory action of Mn^{2+} and Hg^{2+}, and activation by citrate and tartarate ions is not clear. However, it is apparent that activation of APase II and APase III by citrate and tartarate ions was not due to the chelating action of these anions on Hg^{2+} and Mn^{2+}. On the basis of the differential response of APase I, APase II and APase III towards various cations and anions, it is possible to distinguish between the various forms of APases by using appropriate combinations of cations or anions.

4.9.5 Identification of amino acids present near/at the active site

Inhibition of the purified APase isoenzymes by Rose-bengal, a histidine specific dye, suggests the presence of histidine residues at the active sites of the isoenzymes (Table XII). The inhibition of PM-APases by Rose-bengal was found to be competitive in nature (Fig 14) indicating that it competes with the substrate for the active site. The inhibitory action of Rose-bengal was overcome by the addition of pNPP. Of the three isoenzymes, APase I seems to contain the maximum number of histidine residues since its activity was maximally inhibited (87.5%) followed by APase III (70%) and APase II (43.4%).

Free sulfhydryl groups (-SH groups) were also found to be associated with the active sites of APase I and APase II since both the isoenzymes were inhibited by iodoacetate and iodoacetamide, the reagents that alkylate the -SH groups (Table XII). Inhibition was overcome by the addition of substrate. However, no -SH groups were involved with the active site of APase III as indicated by its lack of inhibition by either of the two reagents. It, thus, appears that

TABLE XII

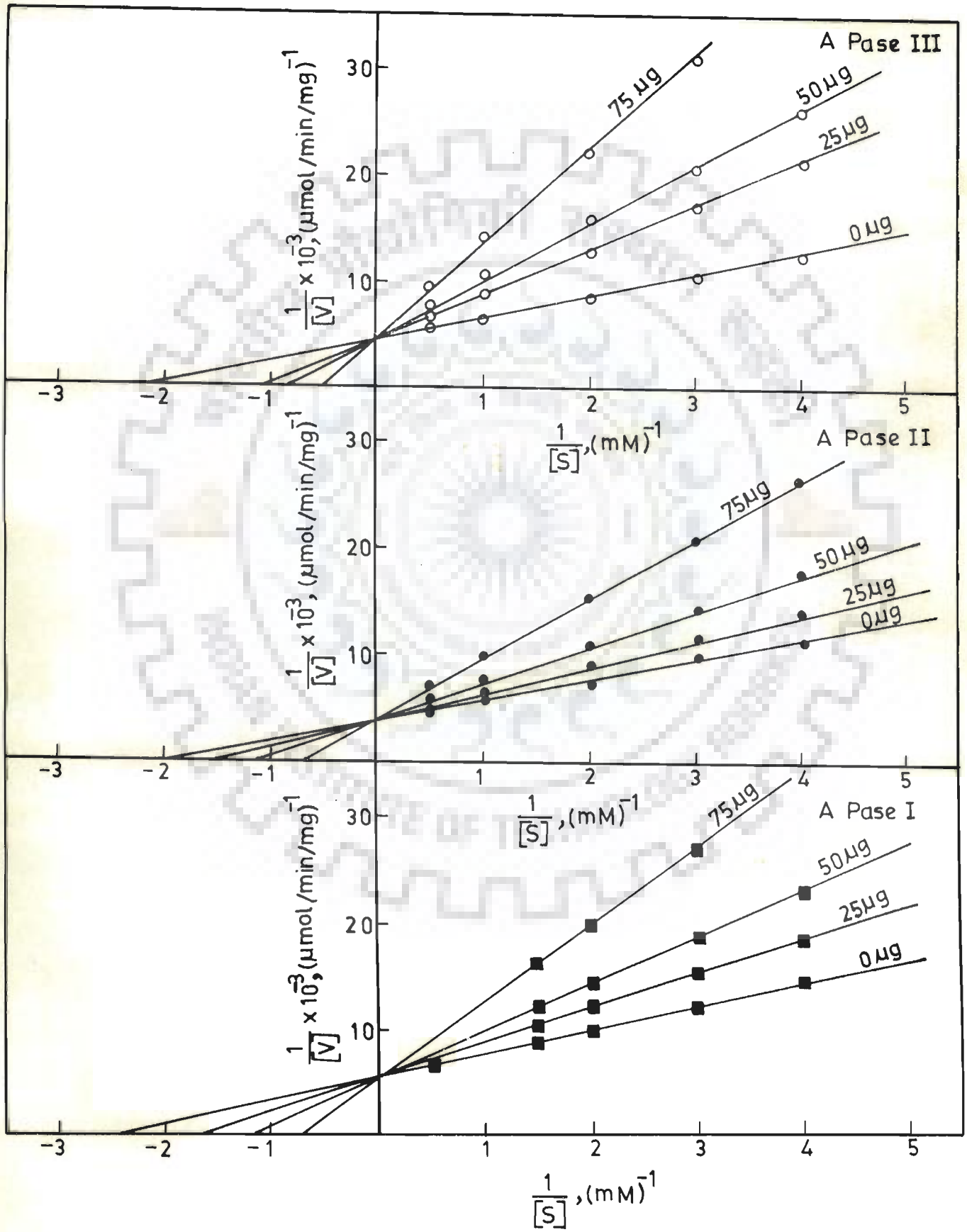
EFFECT OF SOME CHEMICAL INHIBITORS ON THE ACTIVITY OF ACID PHOSPHATASE ISOENZYMES

The purified APase isoenzymes were incubated with the chemical inhibitors at pH 5.0 and 4°C for 30 min, after which the APase activity was assayed in a normal manner. Control reaction mixtures were without the inhibitors. Enzyme activities were represented as percent of residual activity with respect to the control, whose activity was considered to be 100%.

Chemical inhibitor	Residual activity (%) (Inhibitor + Enzyme)			Residual activity (%) (Inhibitor + Substrate + Enzyme)		
	APase I	APase II	APase III	APase I	APase II	APase III
Rose-bengal (50 µg)	12.5	56.6	30.0	90.2	85.9	72.5
Iodoacetate (2 mM)	62.5	71.9	100	100	100	100
Iodoacetamide (2 mM)	58.2	64.3	98.2	95.2	100	98.2
Control	100	100	100	100	100	100

FIGURE 14

Lineweaver-Burk plots of purified acid phosphatase isoenzymes showing inhibition by Rose-bengal. *Enzyme assays were carried out in a normal manner with varying amounts of the substrate p-nitro phenyl phosphate (0.25 mM - 2 mM) in the presence of 0, 25, 50 and 75 µg of Rose bengal. All the three APase isoenzymes exhibited competitive type of inhibition with Rose bengal.*


A Pase III
75 µg
50 µg
25 µg
0 µg
$\frac{1}{[V]} \times 10^{-3}$, (µmol/min/mg)$^{-1}$
$\frac{1}{[S]}$, (mM)$^{-1}$
A Pase II
75µg
50µg
25µg
0µg
$\frac{1}{[V]} \times 10^{-3}$, (µmol/min/mg)$^{-1}$
$\frac{1}{[S]}$, (mM)$^{-1}$
A Pase I
75µg
50µg
25µg
0µg
$\frac{1}{[V]} \times 10^{-3}$, (µmol/min/mg)$^{-1}$
$\frac{1}{[S]}$, (mM)$^{-1}$

cysteine residues are involved with the active sites of APase I and APase II but not APaase III.

4.9.6 Glycoprotein nature

All three APase isoenzymes were found to be glycoproteins with 50% (APase I), 27% (APase II) and 30% (APase III) carbohydrate as estimated by the phenol/H_2SO_4 acid method (46).

4.9.7 Effect of sodium meta periodate

Sodium metaperiodate treatment of the APase isoenzymes resulted in a sharp decrease in the activities of APase I, APase II and APase III as shown in Fig 15. More than 50% of the original activity was lost within 10 min of reaction in all three cases which further decreased to less than 20% after 60 min of reaction. The results indicate that the carbohydrate moieties of the APase isoenzymes are essential for maintaining them in their active forms.

4.9.8 Analysis of monosaccharides by HPLC

Table XIII shows the analysis of sugars present in purified APase isoenzymes by HPLC. APase I was found to contain D-man and GlcNAc (Fig.16) while APase II and APase III were mainly found to contain D-man and D-glu as the main sugars (Fig.17 and Fig.18). Treatment of APase I with endo-N-acetyl-β-D-glucosaminidase, which cleaves the glycosidic bond between two adjacent N-acetyl glucosamine residues releases almost total carbohydrate present in APase I but does not release the carbohydrates of APase II and APase III. The nature of peptide-carbohydrate linkage of APase I, therefore, appears to be N-glycosidic in nature. Absence of GlcNAc and the inability of Endo-H to release the oligosaccharides of APase II and APase III suggests O-glycosidic linkages and/or complex type of carbohydrate in APase II and APase III isoenzymes.

4.9.9 Identification of associated metals

The APase isoenzymes showed the presence of considerable amounts

FIGURE 15

Effect of sodium metaperiodate on the activities of purified acid phosphatase isoenzymes. *The purified APase I (O--O), APase II (●--●) and APase III (▲--▲) isoenzymes were incubated with 10 mM sodium metaperiodate in dark at 4°C for various time periods. Sodium metaperiodate solution was prepared in 0.1 M sodium acetate buffer (pH 5.0). The reactions were terminated at various time intervals by adding ethylene glycol to a final concentration of 10%. The terminated reaction mixtures were dialyzed 6 h at 4°C against chilled 50 mM sodium acetate buffer (pH 5.0) to remove the excess ethylene glycol and enzyme activities assayed in a normal manner. Control reaction mixtures were devoid of sodium metaperiodate. Enzyme activities were represented as percentage of maximum activity with respect to control whose activity was considered to be 100%.*

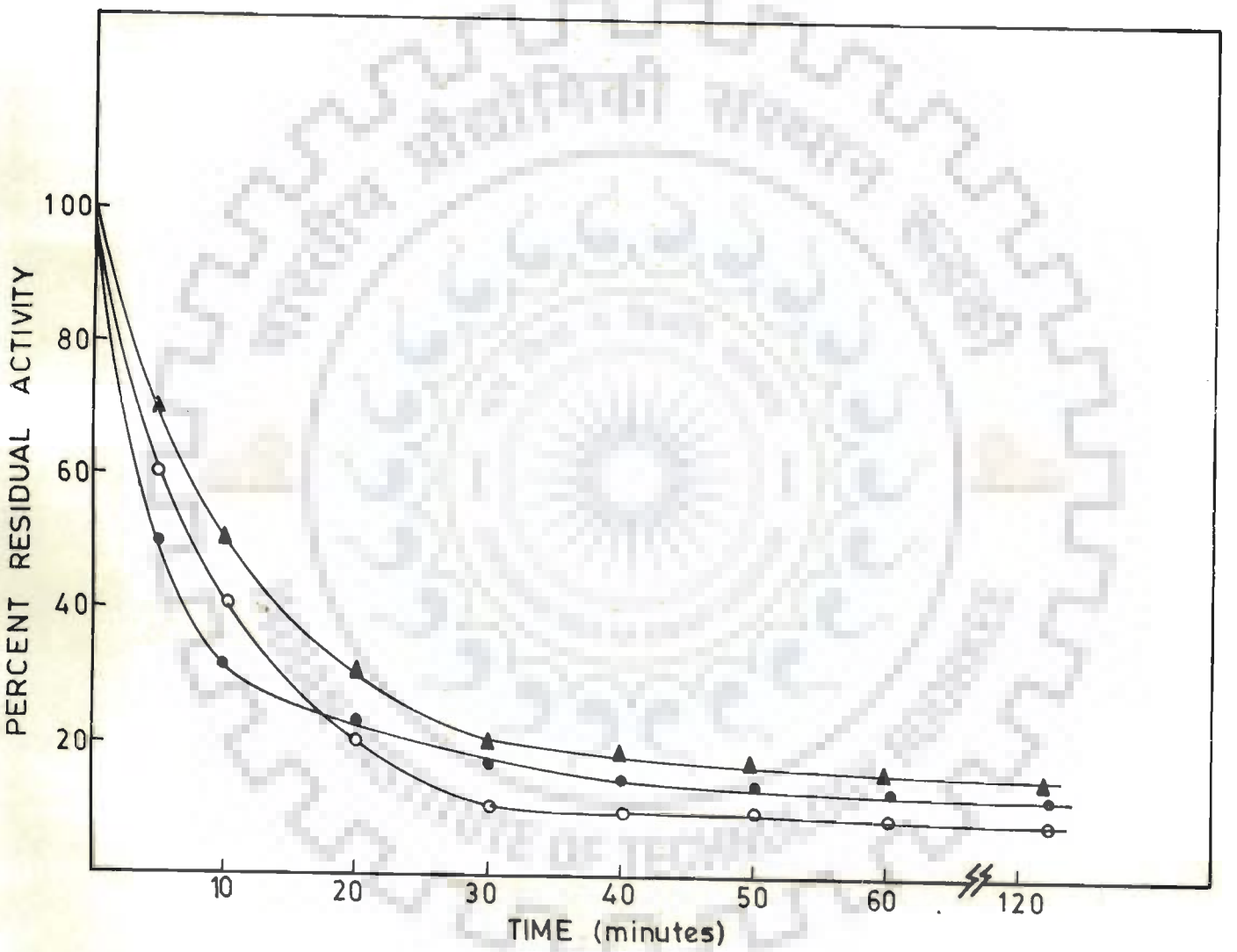

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
10
20
30
40
50
60
120
TIME (minutes)

TABLE XIII

ANALYSIS OF MONOSACCHARIDES BY HPLC

Hydrolyzed glycoprotein samples, prepared as described in Experimental Procedures, were injected on Shimadzu LC-4A system on Zorbax C-18 column. The fractionation was performed with the mobile phase of acetonitrile : water (80:20, v/v) at a flow rate of 0.8 ml/min. The monosaccharide units were detected at 250 nm wavelength.

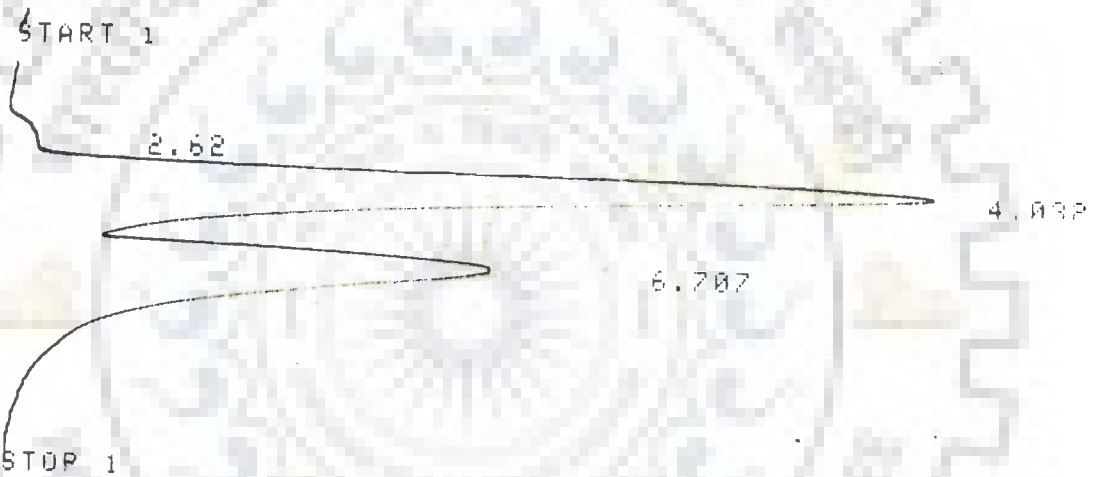
Sugar	Retention time (min)[a]			Standard sample
	APase I	APase II	APase III	
D-Man	4.03 (55.7%)	3.99 (8.8%)	3.85 (11.5%)	3.83
GlcNAc	6.70 (42.9)	-	-	6.88
D-Glu	-	7.27 (91.1%)	7.25 (86.1%)	7.25
D-Gal	-	-	-	7.06

[a] Values in parentheses indicate the percentage of sugar present.

FIGURE 16

Elution profile of monosaccharides present in APase I from the Zorbax C-18 column of HPLC. *The glycoprotein samples were hydrolyzed and derivatized as described under Experimental Procedures. Sample (5 µl) was injected to a 25 cm, 5 µm Zorbax C-18 column of Shimadzu LC-4A HPLC system. Elution was performed with a mixture of $CH_3CN:H_2O$ in a ratio 80:20 for 15 min at a flow rate of 0.8 ml/min. The sugar peaks were detected by the uv spectrophotometric detector at 250 nm wavelength. The monosaccharides of the glycoprotein enzyme were identified by comparing their retention times with those of standard sugars.*

CLOSE PRINTER
BPLOT

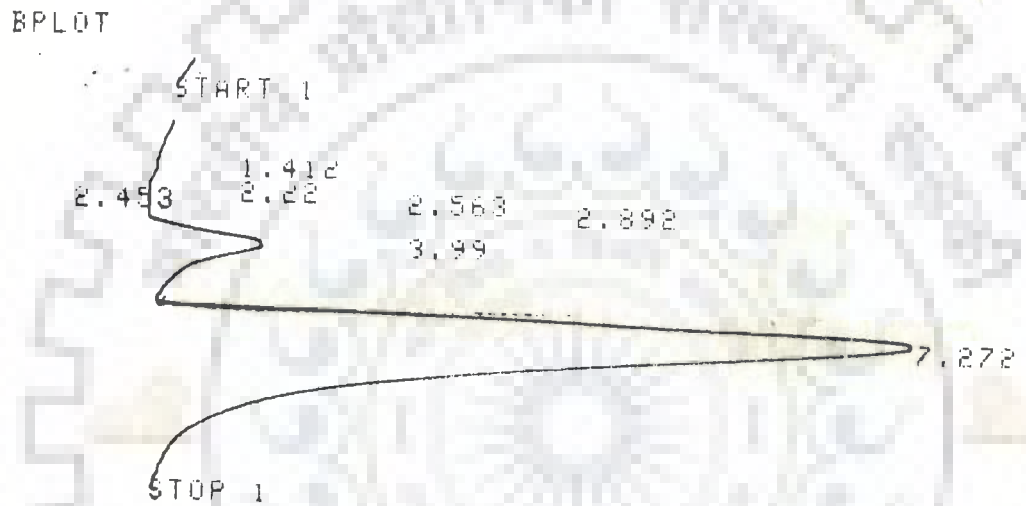


PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.62	107482			1.3801	
2	4.032	4338685	V		55.711	
3	6.707	3341678	V		42.9089	
	TOTAL	7787845			100	

CLOSE PRINTER

FIGURE 17

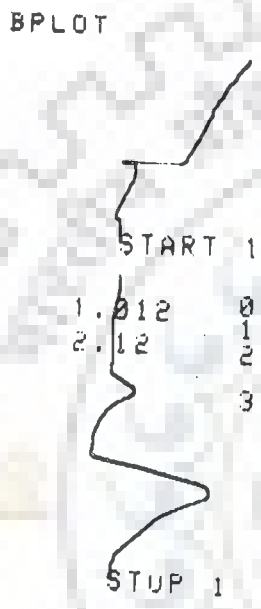
Elution profile of monosaccharides present in APasae II from the Zorbax C-18 column of HPLC. *Conditions for the detection of monosaccharide peaks were exactly the same as described in the legend of FIGURE 16.*



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.99	517203	V		8.8498	
2	7.272	5332543	V		91.1507	
	TOTAL	5850246			100	

FIGURE 18

Elution profile of monosaccharides present in APase III from the Zorbax C-18 column of HPLC. *Conditions for the detection of monosaccharide peaks were exactly the same as described in the legend of FIGURE 16.*



PKNO	TIME	AREA	MK	TYPE	CONC	NAME
1	2.12	12391	V		1.4416	
2	2.38	7820	V		0.9097	
3	3.855	98911			11.5069	
4	7.253	740456			86.1418	
	TOTAL	859578			100	

of Mn and little amounts of Zn as detected by atomic absorption spectroscopy (Table XIV). APase III was found to contain maximum amount of Mn (1.55 µg/g) followed by APase II (1.02 µg/g) and APase I (0.62 µg/g). The presence of Cu and Fe was not detected in any of the APase isoenzymes. Mn and Zn were assumed to be tightly attached to the APase isoenzyme molecules since they were detected even after repeated dialysis against 2 mM EDTA.

4.9.10 Effect of monensin

Table XV describes the effect of monensin on the intracellular levels of APase activity. It was found that total as well as specific activities of APase in the microsomal membrane fraction containing ER, GA and PM from both monensin-treated and untreated cotyledons were comparable. However, the APase activity in the PM from the former (28 µM/min/mg) was significantly lower than the latter (43.4 µM/min/mg). In contrast, the APase activity in GA from the monension-treated cotyledons (57.3 µM/min/mg) was nearly 3-fold higher than that of the control (17.7 µM/min/mg). Thus, it appears that monensin treatment brings about a decrease in the activity of APase in the PM fraction with a concomitant increase in the GA fraction.

4.9.11 Effect of gibberellic acid

The effect of gibberellic acid (GA_3) on the subcellular levels of APase is summarized in Table XVI. It is seen that at a 10^{-4} M concentration of GA_3, there is a substantial rise in the levels if both total activity and specific activity of APase in the PM fraction of GA_3-treated cotyledons as compared to the untreated (control) cotyledons. On the other hand the level of total activity of APase in the GA fraction falls to almost half (6.2 µM/min) in the Ga_3-treated cotyledons as compared to that of the control (12.0 µM/min). It, thus, seems that GA_3 treatment brings about an accumulation of APase activity in the plasma

TABLE XIV

IDENTIFICATION OF ASSOCIATED METALS BY ATOMIC ABSORPTION SPECTROSCOPY

The purified APase isoenzymes were repeatedly dialyzed against deionized double-distilled water and the presence of various metals detected by atomic absorption spectroscopy on an Instrumentation Laboratory Inc. spectrometer. The amount of detected metal was represented as µg metal/g sample protein.

Sample	Metal[a] (µg/g protein)			
	Mn	Zn	Cu	Fe
APase I	0.623	0.094	0.00	0.00
APase II	1.026	0.045	0.00	0.00
APase III	1.552	0.052	0.00	0.00
Control[b]	0.00	0.00	0.00	0.00

[a] The absorption wavelengths of Mn, Zn, Cu and Fe were 279.5 nm, 213.9 nm, 324.7 nm and 248.3 nm, respectively.

[b] Deionized double-distilled water was used as control.

TABLE XV

EFFECT OF MONENSIN ON THE SUBCELLULAR LEVELS OF APase ACTIVITY IN PEANUT COTYLEDONS

Peanut cotyledons were germinated in presence of 1 mM monensin and used to prepare PM, ER and GA fractions as described in Experimental Procedures. Membrane fractions prepared from the normally germinated cotyledons (without monensin) served as controls. The peanut cotyledons were germinated for 48 hours under identical conditions, simultaneously.

	Control (without monensin)			Monensin treated		
Fraction	Total protein (mg)	Total activity (µmol /min)	Specific activity (µmol/min/mg)	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)
Crude microsomal membrane fraction	398.4	8296	20.8	407.2	8038	19.7
Plasma membrane	104.0	4520	43.4	80.0	2240	28.0
Smooth endoplasmic reticulum	64.2	1604	24.9	60.4	1536	25.4
Golgi apparatus	56.6	1004	17.7	59.4	3404	57.4

TABLE XVI

EFFECT OF GIBBERELLIC ACID ON THE SUBCELLULAR LEVELS OF APase IN PEANUT COTYLEDONS

Peanut cotyledons were grown in the presence of 10^{-4}M gibberellic acid (GA_3) for 48 hours and various subcellular fractions prepared as described in Experimental Procedures. Peanut cotyledons grown in the absence of GA_3 under identical conditions served as controls. Values of total APase activity and total protein content were represented per gram fresh weight of cotyledons.

	Control (without GA_3)			GA_3 treated (10^{-4}M)		
Fraction	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)
Crude microsomal membrane fraction	5.99	132	22.0	5.22	128.0	24.5
Plasma membrane	1.38	76.5	55.4	1.14	101.9	89.3
Smooth endoplasmic reticulum	0.99	24.5	24.7	0.85	11.2	13.17
Golgi apparatus	0.48	12.0	25.0	0.31	6.2	19.4
Cytosol	44.0	1288	29.2	49.97	1856.5	37.1

membranes and a depletion of APase activity in the ER and GA fractions of peanut cotyledon cells.

4.9.12 Effect of storage

APase I, APase II and APase III appeared to be quite stable when stored at pH 5.0 and -20°C and retained more than 80% of their original activities even after two weeks of storage (Table XVII).

4.9.13 Thermal properties

Fig. 19 shows the thermal stability of purified APase isoenzymes. As is evident from Fig.19, APase I, APase II and APase III are quite stable upto 50°C and lose 50% of their original activity only after 65°C. Fig. 20 shows the activity of purified APases as a function of temperature. The optimum-temperature range of all three APase isoenzymes spans over a fairly wide range of 30°C to 45°C.

4.9.14 **Immunological properties**

4.9.14.1 Immunological relationship among APase I, APase II and APase III isoenzymes

The PM-APase isoenzymes were found to be immunologically related to each other since all of them cross-reacted with antisera raised against each of the purified isoenzyme. Results have been shown in Fig. 21 (21A, 21B, 21C). Table XVIII summarizes the immunoprecipitation of APase activity by anti-APase I anti-APase II and anti-APase III from purified APase I, APase II and APase III fractions. Anti-APase I immunoprecipitated 33.8% and 27.6% APase activity from APase II and APase III; Anti-APase II precipitated 31% and 35.1% activities from APase I and APase III while Anti-APase III precipitated 25% and 39% APase activities from APase I and APase II, respectively. The three PM-APase isoenzymes, thus, seem to share some common antigenic determinants with each other.

TABLE XVII

STABILITY OF PURIFIED PEANUT COTYLEDON PM-APase ISOENZYMES AT -20°C

Purified APase I, APase II and APase III isoenzymes were stored at pH 5.0 for varying time periods at -20°C and then assayed in a routine manner as described in Experimental Procedures.

Period of storage (days)	Residual APase activity (%)		
	APase I	APase II	APase III
0	100	100	100
1	98.3	95.0	97.0
2	92.0	90.1	95.8
4	90.4	89.5	93.2
8	88.2	85.0	89.7
10	85.6	84.8	86.8
14	82.0	84.0	85.3

FIGURE 19

Thermal stability of purified acid phosphatase isoenzymes. *Purified APase I (O--O), APase II (●--●) and APase III (▲--▲) were kept at 50°C for the indicated periods of time after which their activities were assayed in a normal manner. Enzyme activities were expressed in terms of percent residual activity with respect to control whose activity was considered to be 100%.*

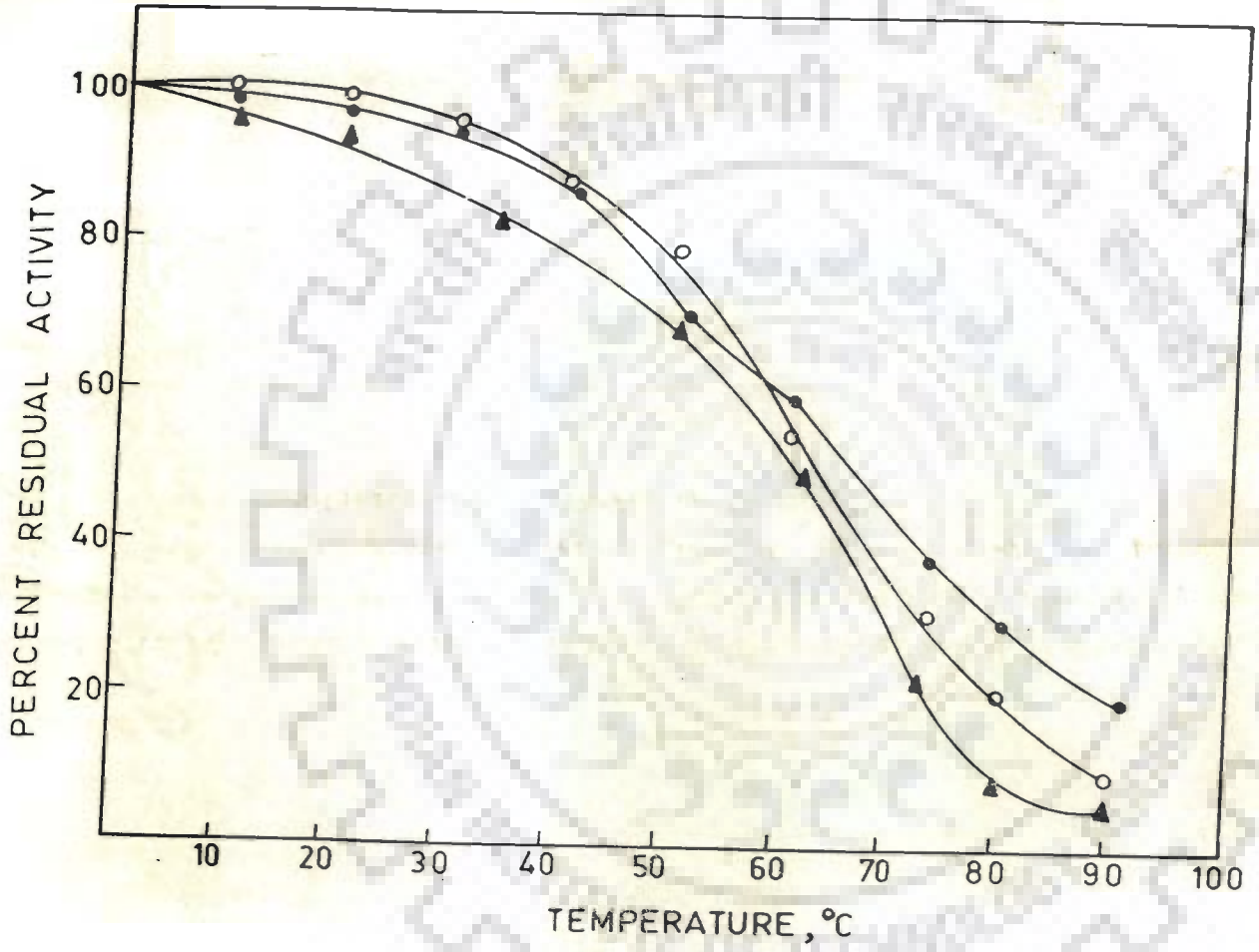

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
10
20
30
40
50
60
70
80
90
100
TEMPERATURE, °C

FIGURE 20

Temperature-activity profiles of purified acid phosphatase isoenzymes. *The activities of purified APase I (O--O), APase II (●--●) and APase III (▲--▲) were assayed at various temperatures ranging from 20°C to 60°C. Controls were run simultaneously under identical conditions. Enzyme activities were expressed in terms of total acid phosphatase activity measured at the indicated temperatures.*

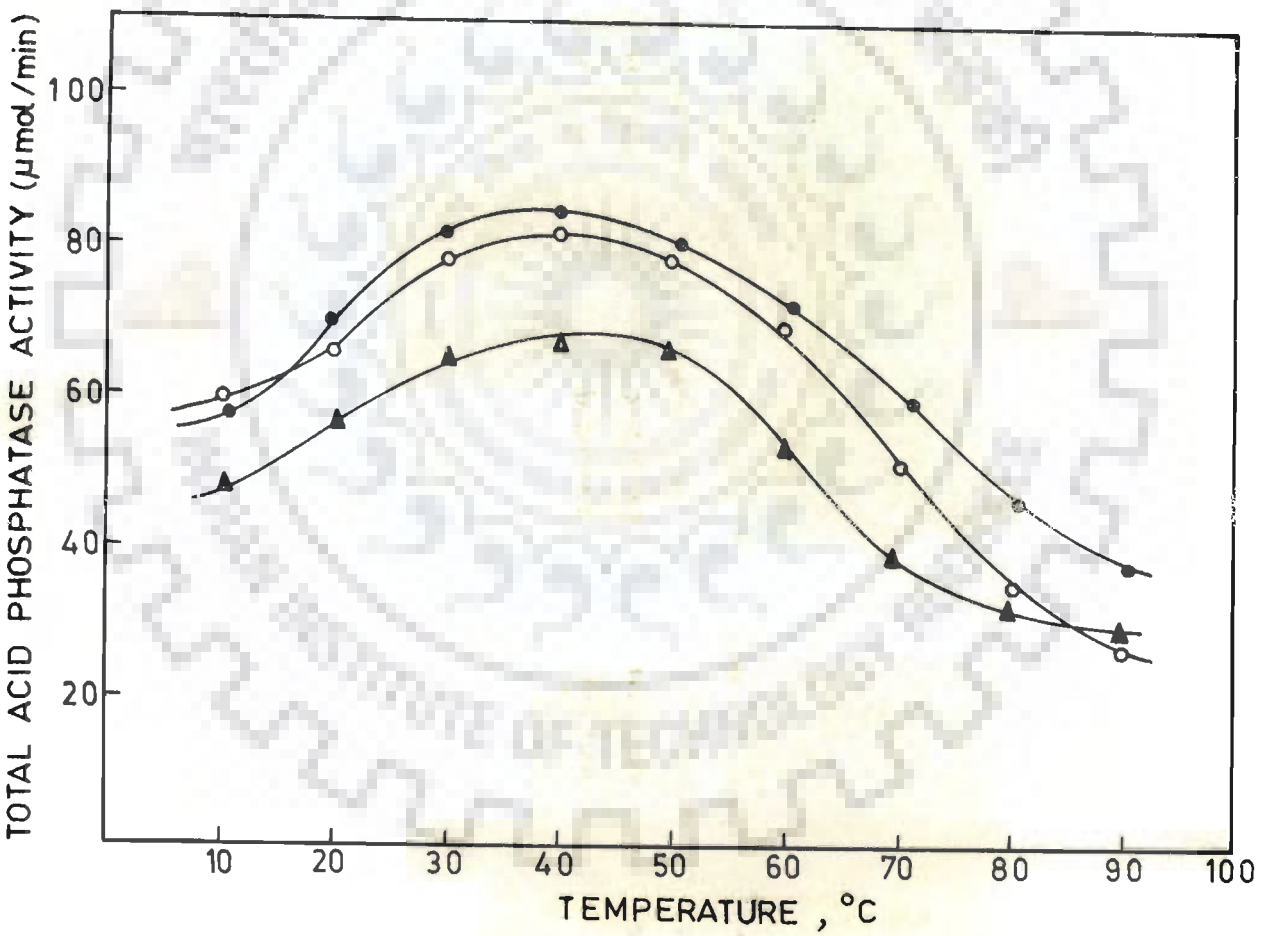

TOTAL ACID PHOSPHATASE ACTIVITY (µmol/min)
100
80
60
40
20
10
20
30
40
50
60
70
80
90
100
TEMPERATURE , °C

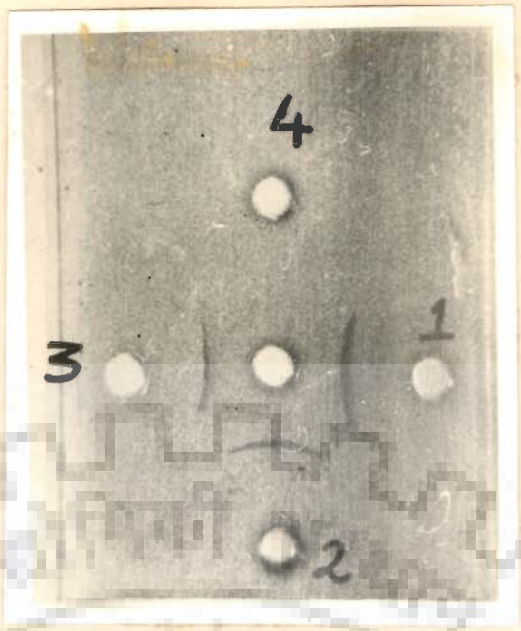
FIGURE 21

Immunodiffusion patterns of purified acid phosphatase isoenzymes of peanut cotyledon plasma membranes with anti-APase I, anti-APase II and anti-APase III. *Conditions employed for the immunodiffusions were those as described under Experimental Procedures. The precipitin bands were stained with 1% Coomassie Brilliant blue for 30 min and destained with 10% acetic acid. The central wells were filled with 10 µl (10 µg protein) of each of the purified glycoprotein isoenzyme. Wells numbered 1, 2 and 3 were filled with antisera at a dilution of 2X while the well number 4 was filled with 8 µl of preimmune sera which served as control.*

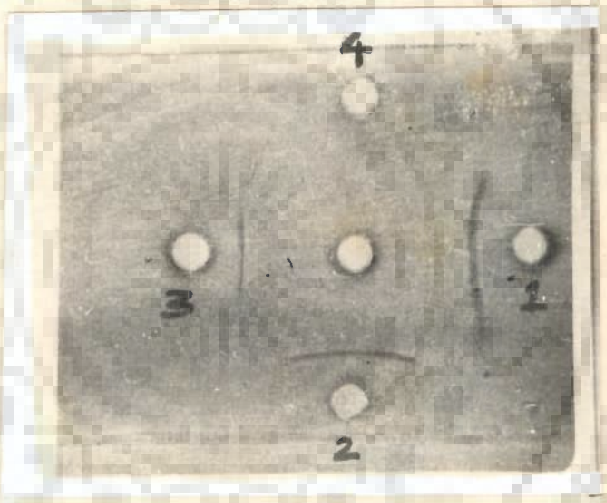
Figure 21A : Central well contained 10 µl (10 µg) of purified APase I; well no.1 contained anti-APase I (8 µl); well no.2 contained anti-APase II (8 µl); well no.3 contained anti-APase III (8 µl); well no.4 contained preimmune sera (8 µl).

Figure 21B : Central well contained 10 µl (10 µg) of purified APase II; well no.1 contained anti-APase I (8 µl); well no.2 contained anti-APase II (8 µl); well no.3 contained anti-APase III (8 µl); well no.4 contained preimmune sera (8 µl).

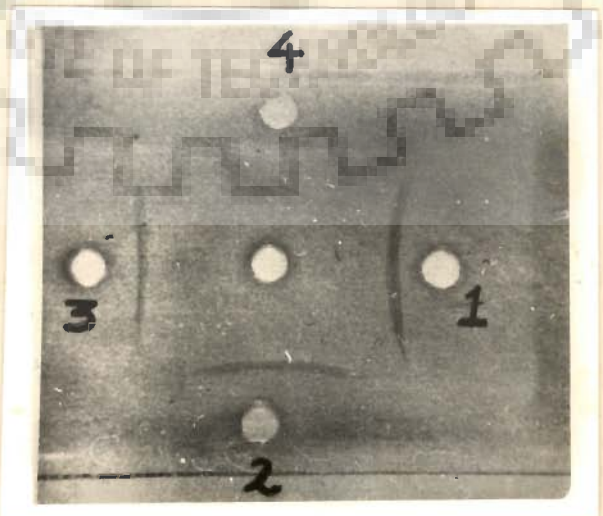
Figure 21C : Central well contained 10 µl (10 µg) of purified APase III; well no.1 contained anti-APase I (8 µl); well no.2 contained anti-APase II (8 µl); well no.3 contained anti-APase III (8 µl); well no.4 contained preimmune sera (8 µl).



21A



21B



21C

TABLE XVIII

IMMUNOPRECIPITATION OF APase ACTIVITY FROM THE PURIFIED APase ISOENZYMES BY ANTI-PM-APases

The immunoprecipitation of APase activity by anti-APase I, anti-APase II and anti-APase III from purified PM-APases was carried out as described in Experimental Procedure. Control 1 contained pre-immune sera while control 2 did not contain any antisera. Amount of immunoprecipitation was expressed in terms of percent residual activity remaining in the supernatant fluid with respect to control whose activity was considered to be 100%.

Fraction	Residual activity (%)				
	Anti-APase I	Anti-APase II	Anti-APase III	Control 1	Control 2
APase I	8.2	69.0	75.0	97.2	100
APase II	66.2	9.4	61.0	92.8	100
APase III	72.4	64.9	9.9	91.9	100

4.9.14.2 Immunoprecipitation of APase activity by anti-APase I, anti-APase II and anti-APase III from the PM, GA and ER fractions of peanut cotyledons

Anti-APase I, anti-APase II and anti-APase III were seen to immunoprecipitate APase activity from PM, GA and ER membrane fractions of peanut cotyledons to varying degrees. Results are summarized in Table XIX. The degree of precipitation of APase activity by all the three anti-APases was maximum in the PM fraction, followed by GA and ER fractions, respectively.

4.9.14.3 Immunological relationship of PM-APase isoenzymes of peanut cotyledons with a PM-APase isoenzyme of pea cotyledons and epididymal APase of Ram

The peanut cotyledon PM-APase isoenzymes were found to be immunologically related to AP-I isoenzyme of pea cotyledon PM (section 3.2.12.1.1) since anti-APase I, anti-APase II and anti-APase III of peanut cotyledon PM were found to cross-react with AP-I isoenzyme (Fig. 22A). However, no cross-reactivity of peanut cotyledon anti-APases was seen with the AP-II isoenzyme of pea cotyledon PM as is evident from Fig 22. Results are summarized in Table XX.

APase present in the tissues of Ram epididymis cross-reacted with anti-APase I, anti-APase II and anti-APase III (Fig 22C), suggesting that peanut cotyledon PM-APases share common epitopes with AP-I isoenzyme of pea cotyledon PM and Ram epididymal APase.

TABLE XIX

IMMUNOPRECIPITATION OF APase ACTIVITY FROM THE PM, GA AND ER FRACTIONS OF PEANUT COTYLEDONS BY ANTI-PM-APases

The PM, GA and ER membrane fractions were prepared and solubilized by 0.5% n-octylglucoside as described in the Experimental Procedures. The solubilized PM, GA and ER membrane fractions were added to the antisera raised against the purified PM-APase isoenzymes as described in the text and kept for immunoprecipitation at 4°C for one week. The amount of immunoprecipitation was expressed in terms of percent residual activity with respect to the control whose activity was considered to be 100%. No antisera was added to control 1 whereas preimmune sera was added to control 2.

Fraction	Residual activity (%)				
	Anti-APase I	Anti-APase II	Anti-APase III	Control 1	Control 2
Solubilized PM	46.4	37.0	51.2	100	98.9
Solubilized GA	60.8	54.8	64.6	100	94.2
Solubilized ER	68.1	60.9	74.1	100	93.5

FIGURE 22

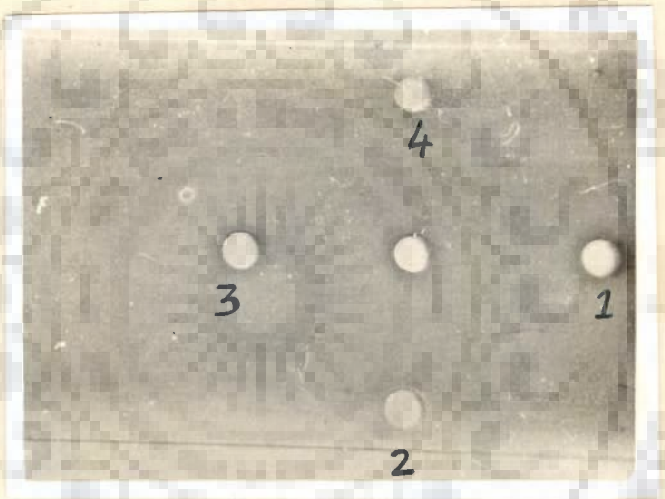
Immunodiffusion patterns of pea cotyledon PM-APase isoenzymes and ram epididymal APase with antisera raised against purified PM-APase isoenzymes of peanut cotyledons. *Conditions for immunodiffusion were those described in the text. Wells numbered 1,2 and 3 were filled with 8 µl each of undiluted antisera while well no.4 was filled with 8 µl of preimmune sera which served as control.*

Figure 22A : Central well contained 10 µl (10 µg) of AP-I isoenzyme of pea cotyledon PM; well no.1 contained anti-APase I; well no.2 contained anti-APase II; well no.3 contained anti-APase III; well no.4 contained preimmune sera.

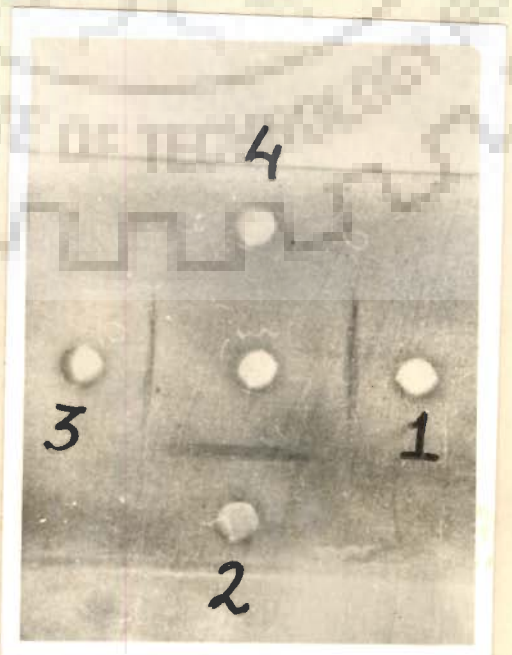
Figure 22B : Central well contained 10 µl (10 µg) of AP-II isoenzyme of pea cotyledon PM; well no.1 contained anti-APase I; well no.2 contained anti-APase II; well no.3 contained anti-APase III; well no.4 contained preimmune sera.

Figure 22C : Central well contained 10 µl (10 µg) of partially purified ram epididymal APase; well no.1 contained anti-APase I; well no.2 contained anti-APase II; well no.3 contained anti-APase III; well no.4 contained preimmune sera.

22A



22B



22C

TABLE XX

IMMUNOPRECIPITATION OF APase ACTIVITY BY ANTI-APases OF PEANUT COTYLEDON PM FROM THE PEA COTYLEDON PM-APases AND RAM EPIDIDYMAL APase.

Immunoprecipitation of APase activity from the AP-I and AP-II isoenzymes of pea cotyledon PM and ram epididymal APase by anti-PM-APases of peanut cotyledon cells was carried out as described in Experimental Procedure. Control 1 did not contain any antisera while control 2 contained preimmune sera. Degree of immunoprecipitation was expressed in terms of percent residual activity with respect to control whose activity was considered to be 100%.

Fraction	Residual activity (%)				
	Anti-APase I	Anti-APase II	Anti-APase III	Copntrol 1	Control 2
Pea cotyledon PM-APases					
AP-I	60.7	54.2	49.3	100	97.2
AP-II	98.2	98.0	95.6	100	98.0
Ram epididymal APase	75.2	68.4	70.8	100	95.2

5.0 *DISCUSSION*

The purpose of the present work was to isolate and characterize the acid phosphatase isoenzymes from the plasma membranes of peanut cotyledons and to investigate the possibility of using acid phosphatase as a model glycoprotein for studying the intracellular transport of proteins in plant storage cells. Although acid phosphatase (APase) was present in the ER, GA and PM fractions of peanut cotyledons, we chose to purify APase present in the PM fraction because more than 66% of the total precipitable APase activity was found to be associated with this membrane fraction. The PM fraction, obtained by the method described in section 3.2.2.2, was of high purity as judged by the high specific activity of glucan synthetase II, a marker enzyme for the plant plasma membranes (70,163) and absence of glucan synthetase I activity, a marker enzyme for GA (65). Since the PM fraction was prepared in the presence of EDTA and without Mg^{2+} ions, the membrane fraction was also considered to be free from rough ER, a common contaminant of PM (70). Furthermore, the presence of EDTA is also reported to maintain the integral structure of the PM (70).

The isoenzymic pattern of peanut cotyledon PM-APases was found to change with germination period as reflected by changes in relative levels of activities of the APase isoenzymes. Such phenomenon is not uncommon in plant APases and similar observations have been reported in APases from germinating maize scutellum (197) and germinating pea cotyledons (90). It, thus, appears that the number and relative levels of activity of APase isoenzymes present in the PM fractions of Arachis hypogaea cotyledons are dependent upon the physiological state of the cotyledons. It is possible that modification of PM-APase isoenzymes occurs during germination and that these modifications play a role in the development of peanut seedlings as has been suggested in the case of maize scutellum APase

(197).

For the purpose of purification of PM-APases we used 2-days old peanut cotyledons becase at this stage of germination, interference of 5'-nucleotidase in the purification of PM-APases was avoided since 5'-nucleotidase activity emerged only after two days of imbibition. This step was thought to be essential because one of the major difficulties was the separation of 5'-nucleotidase from the APase as the two enzymes have similar solubilizing characteristics (181) and in general APases exhibit 5'-nucleotidase activity (83). Further, even the apparently pure 5'-nucleotidase preparation (homogeneous by SDS-PAGE) was found to show APase activity as well (181,31). Our results show that by choosing germinating cotyledons of appropriate age, interference of 5'-nucleotidase in the purification of PM-APases was totally avoided.

The treatment of PM fraction with n-octylglucoside in the presence of Mg^{2+} and EDTA at a protein-to-detergent ratio of 2:3 resulted in a selective and true solubilization of PM-bound APases since the enzyme remained soluble, stable and active even after the removal of the detergent. Under these conditions, a 6.6 fold purification with 31% yield was achieved. Column chromatography on CM-Sephadex and Sephadex G-150 columns resulted in the separation of three distinct APase isoenzymes, designated as APase I, APase II and APase III in the order of their elution.

All the three APase isoenzymes exhibited a pH-optimum at pH 5.0. The striking feature of these APase isoenzymes was that all of them were found to exist in monomeric forms at pH 5.0 which were subsequently converted to dimeric forms when exposed to pH 7.2. The dimeric forms again exhibited a monomeric character when the pH was lowered from pH 7.2 to pH 5.0. The pH stability data shows that PM-APases are more stable at pH 5.0 than at pH 7.2. It, therefore, appears that

APases in monomeric forms are more stable than the dimeric forms. The pH-dependent association - dissociation phenomenon exhibited by the PM-APase isoenzymes may provide the mechanism by which the activity of APases is regulated in plant cells, particularly in the storage cells, during germination when large pH variation is likely to occur. Since the physiological pH of the intracellular environment is generally around 7.0, it is likely that acid phosphatase occurs as a dimer *in vivo*.

APase I, APase II and APase III were found to be glycoproteins with 50%, 27% and 30% carbohydrate, respectively. In this respect they confirm the general glycoprotein nature of plant APases (36,55,60,85,95,119,152,197,182,213). Inhibition of APase I and APase II by orthophosphate in a competitive manner is also in agreement with other similar reports (55,63,95,152,167,182,197). It is suggested that inhibition of PM-APase isoenzymes by orthophosphate may be involved in the regulation of APase activity in the PM of germinating peanut cotyledons. The broad range of thermal stability of APase I, APase II and APase III follows the general pattern of resistance of plant APases to thermal inactivation at high temperatures (35,85,95,190).

The substrate specificities of the APase isoenzymes was found to be pH-dependent with degree of specificity for each isoenzyme being significantly different from the other. Since at pH 7.2, the APase isoenzymes exhibit greater substrate specificity towards nucleotide phosphates and sugar phosphates as compared to at pH 5.0, it appears that PM-APases in germinating peanut cotyledon cells are capable of actively hydrolysing a vast array of available substrates as the intracellular pH is generally around 7.0. This may also be essential for the growing seedling because during germination the level of APase goes up in the cells and consequently more amount of substrate is required. By hydrolysing a variety of sugar-and nucleotide phosphates, the increased substrate

demands of the growing levels of APases are met more easily.

APase isoenzymes showed highly differential response towards various cations and anions. The differential response of the APase isoenzymes clearly demonstrates that the three isoenzymes are different from each other and are not the modified or proteolytic products of the same enzyme. All the three APases were found to be metalloproteins containing significant amounts of Mn and little amounts of Zn. These metals were found to be tightly bound to the isoenzyme molecules since they could not be separated from the isoenzymes even after extensive dialysis against EDTA. The strong inhibition of APase I by Mn^{2+} ions inspite of the presence of Mn can be explained by the fact that Mn is probably not associated with the active site of APase I but tightly bound to the isoenzyme at sites other than the active site and that association of Mn with these sites has no effect on the catalytic activity of the enzyme molecule. The same arguement also holds true in case of Zn. Also, in all probability, the Mn and Zn are bound so tightly to these sites that any conformational flexibility of these metals is restricted and they have no chance of interacting with the active sites of APases and, thus, have no chance to inhibit them. The presence of Mn in APase I, APase II and APase III is especially interesting because all the other Mn-containing plant APases have been generally found to possess a characteristic violet color (60,85,201). This, perhaps, is the first report of Mn- containing APase isoenzymes from a plant source without the characteristic violet color.

Histidine residues were found to be present near/at the active site of APase I, APase II and APase III. The presence of essential histidine residues has also previously been reported in Mn- containing APase of sweet potato (62). Active sites of APase I and APase II were also found to contain essential -SH groups. In all probability cysteine residues are involved with the active sites

of APase I and APase II. However, APase III did not seem to contain any -SH groups associated with its active site. Although APase II was found to contain cysteine residues at its active site, its inhibition by Hg^{2+} ions was not observed probably due to 'substrate protection' of APase II by p-nitrophenyl phosphate present in the reaction mixture.

High degree of inhibition of APase activity by sodium meta periodate treatment suggests a role for the carbohydrate moieties of APase I, APase II and APase III in maintaining the catalytic activity of the enzyme. Since periodate (IO_4^-) cleaves the bonds between carbon atoms containing adjacent -OH groups, it disrupts the sugar structures of the carbohydrates of the glycoprotein isoenzymes. This disruption was seen to bring about a severe inhibition in the activities of the APase isoenzymes and suggests that the carbohydrate chains of APase I, APase II and APase III are required for maintaining the catalytic activity of the isoenzymes and keeping them in an active conformation.

Carbohydrate analysis of purified APases showed the presence of D-man and GlcNAc as the main sugars in case of APase I while APase II and APase III were mainly found to contain D-man and D-glu. The high - mannose type glycoprotein nature of APase I is similar to the APase (i form) purified from the mycelia of <u>ASpergillus niger</u> that was found to contain 89% D-man of the total neutral sugar content (182). The repressible APase from yeast is also reported to contain more than 50% mannose (128). High glucose content of APase II and APase III is not very surprising since APases from different sources have been found to contain high amounts of glucose (88,119).

The nature of peptide-carbohydrate linkage seems to be N-glycosidic in APase I with almost complete certainity since almost the whole of the carbohydrate could be released by endo-N-acetyl-β-D-glucosaminidase treatment. On the other

hand, the carbohydrate moieties of APase II and APase III do not appear to be N-glycosidically linked since Endo-H treatment could not bring about a release of their carbohydrate moieties and also because their sugar analysis show the total absence of GlcNAc. The high glucose content of APase II (91%) and APase III (86%) suggests that glucan chains may be attached through O-glycosidic linkages with serine or threonine residues of polypeptide chains of APase II and APase III. This is an interesting observation since plant APases are generally known to possess N-glycosidic linkages and O-glycosidic linkages are rare among plant APases.

It was found that in the presence of monensin the level of PM-APase decreased while the level of GA-APase increased significantly compared to the control Since monensin is thought to disrupt the traffic of proteins within plant cells (24), it seems that monensin blocks the intracellular transport of APase from GA to PM in peanut cotyledon cells and therefore causes accumulation of APase in the GA. It is generally accepted that proteins destined for export are synthesized on the rough ER, vectorially discharged into the lumen of rough ER, translocated to Golgi in vesicles, move through various lamellae of the Golgi stack and are finally packaged in secretory vesicles for transport to the plasma membranes (145,189). Since monensin treatment disrupts the traffic of APase from GA to PM, the glycoprotein enzyme must pass from GA to PM. It may, thus, be pertinent to assume that APase present in the peanut cotyledon cells must follow the route ER→GA→PM for transport within the cell and must be a useful glycoprotein for studying the intracellular transport.

Gibberellic acid (GA3) treatment brought about an accumulation of APase activity in the PM fraction but a decrease in the APase activities in GA and ER fractions of peanut cotyledon cells as compared to control. This is in contrast

to the situation observed in barley aleurones where GA_3- treatment was clearly seen to increase the APase activity in ER and GA fractions (93). Our results may be explained on the basis of observations made by Jones that the ER of the GA_3-treated barley aleurone cells was seen to be closely associated with the PM at several points as well as with the GA (93). If GA_3-treatment is also presumed to bring about similar kind of direct connections of ER with the PM and if APase is presumed to follow the route ER→GA→PM for intracellular transport, then direct association of ER with the PM would ensure a direct and faster transport of APase from ER to PM with no intermediary passage through GA. The result of this faster channelling would be a relatively high activity of APase in the PM fraction and lesser APase activities in ER and GA fractions in GA_3- treated cotyledons. The lower activities of APase in ER and GA fractions also suggests that enhancement of APase activity by GA_3- treatment is by the activation of preformed APase molecules and not by <u>de novo</u> synthesis. Had <u>de novo</u> synthesis of APase occured, the ER fraction of GA_3- treated cotyledons would have shown an increased level of APase activity since protein synthesis occurs in the ER membranes. This is only a tentative proposition and more detailed experiments are needed for confirmation. The work on these lines is in progress.

Immunological studies lend further support to the theory transport of PM-APases via the route ER → GA → PM. It was reasoned that if APase isoenzymes present in the plasma membranes have to pass through GA and ER as well, then antibodies raised against purified PM-APase isoenzymes should be able to precipitate the APases present in GA and ER also. This hypothesis was put to test by raising antibodies against purified APase I, APase II and APase III from the PM fraction and by immunoprecipitating APase activity from ER and GA fractions along with the PM fraction. Anti-APase I, anti-APase II and anti-APase III immunoprecipitated APase activity from all the three membrane fractions (Table XIX),

thereby, strengthening our proposition that APase in peanut cotyledon cells is intracellularly transported via the route ER→GA→PM. Since all along this route, major changes occur in the carbohydrate moieties of glycoproteins as a result of increasing glycosylation, with the protein core remaining almost unchanged, it may be assumed that the decreasing degree of immunoprecipitation of APase activity from PM to GA to ER by anti-PM-APases (Table XIX) is mainly due to changes in the carbohydrate structures of APases present in these membrane fractions. Based on this assumption it is suggested that the carbohydrate moieties of APase I, APase II and APase III may act as antigenic determinants and that APase isoenzymes present in the PM, GA and ER membranes may each have a set of antigenic determinants in their carbohydrate regions that is membrane-specific. These membrane-specific 'carbohydrate' epitopes would be valuable markers for the identification of various membranes. Monoclonal antibodies raised against these unique membrane-specific determinants would prove to be a powerful and reliable tool in isolating and identifying the PM, GA and ER membrane fractions of peanut cotyledon cells.

APase I, APase II and APase III were found to be immunologically related with each other and, thus, assumed to share some common epitopes. Similar immunological relationship among APase isoenzymes have been reported to occur in the cytoplasmic and membrane-bound ATPases from yam tuber (96) and among APases of various grass species (133). Peanut cotyledon PM-APase isoenzymes were also found to share common epitopic regions with an APase isoenzyme (AP-I) of pea cotyledon PM, thereby, proving that immunological relationship exists between PM-APases of peanut and pea cotyledons. This was not totally unexpected as APases from various plant sources have been known to share common epitopic regions. More surprising, though, was the immunological relationship of APase I, APase II

and APase III with the APase present in the epididymal extract of ram (belongs to goat family). Ram epididymal APase was cross-reacted with anti-APases of peanut cotyledon PM to see whether PM-APases shared any epitopes with APase from an animal source. The affirmative observation suggests an interesting evolutionary hypothesis. Since APases from peanut cotyledon PM, pea cotyledon PM and Ram epididymis share common antigenic determinants, it is possible that during the process of evolution some regions in these APase molecules have remained conserved, as reflected by their common epitopic regions, irrespective of the source (i.e. whether it is from a plant source or an animal source). Possibility also exists that the above mentioned APases have all originated from a common ancestral gene and during the lengthy and dynamic process of evolution have become sufficiently distinct though still possessing some common residual epitopic regions. In a broader sense it would mean that various plant and animal APases have all originated from a common ancestral gene. This is merely a hypothesis that requires detailed studies to carry sufficient weight.

6.0 SUMMARY AND CONCLUSIONS

Plasma membrane-bound acid phosphatase isoenzymes from peanut cotyledon cells have been purified and characterized in the present investigation in an attempt to provide more information regarding plant membrane-bound APases since efforts to purify them have met with only partial success and they still remain poorly characterized to date. The route of intracellular transport of APase and its possible use as a model glycoprotein for studying the intracellular transport of proteins in plant storage cells has also been investigated in our present study.

Highly enriched PM, GA and ER membrane fractions from peanut cotyledon cells were obtained by subjecting the crude microsomal membrane pellet (12,000-105,000 x g pellet) to a series of step sucrose gradient centrifugation that resulted in the separation of the PM, GA and ER fractions as judged by the activities of marker enzymes in these three fractions. The PM fraction was found to be almost free of any GA and ER contamination. The ER fraction also appeared to be virtually free of any PM contamination. The GA fraction exhibited a very high activity of the marker enzyme glucan synthetase I suggesting no significant cross-contamination by either the PM or ER.

APase isoenzymes were purified from the PM fraction because more than 66% of the total precipitable APase activity was found to be associated with this membrane fraction and in terms of specific activity also, the PM fraction showed the maximum APase activity (40.9 µm/min/mg). The isoenzymic pattern of APase in the PM fraction of peanut cotyledons was found to change with progressive germination along with changes in their relative levels. The elution profiles of PM-APase isoenzymes from CM-Sephadex C-50 column indicated

the presence of three APase isoenzymes (APase I, APase II and APase III) from 0-days and 2-days old cotyledons but only two isoenzymes (APase I and APase III) from 7-days old cotyledons with the APase II isoenzyme being absent from the latter. These observations suggest that the number and relative levels of activities of PM-APase isoenzymes are dependent upon the physiological state of the cotyledons.

Changes in the level of APase activity as a function of germination period indicated maximum activity of the enzyme between 6th and 7th day of germination. However, 2-days old peanut cotyledons were used for purifying PM-APases because at this stage of germination, interference of 5'-nucleotidase in the purification of PM-APases was totally avoided since 5'-nucleotidase emerged only after two days of imbibition.

The purification scheme of PM-APases involved only four major steps, namely, preparation of high specific activity PM fraction, solubilization of APases by a suitable detergent, a subsequent ion-exchange chromatography on CM-Sephade C-50 resin followed by gel filtration on Sephadex G-150 resin.

Of the five detergents used (deoxycholate, taurocholate, triton X-100, nonidet P-40 and n-octylglucoside), n-octylglucoside was found to be the best one for solubilizing the PM-APases since under the conditions employed, it solubilized two-third of the total enzyme activity with a 6.7 fold increase in specific activity. Mg^{2+} and EDTA were found to be essential for selective solubilization of APases from the PM. Solubilization of PM-APases was performed with 0.5% n-octylglucoside at a protein-to-detergent ratio of 2:3 in presence of 20 mM $MgCl_2$ and 5 mM EDTA. Even after the removal of the detergent by dialysis, the PM-APases were found to remain in soluble, stable and active form indicating

their true solubilization. Further purification was performed by CM-Sephadex C-50 chromatography that resulted in the separation of three distinct APase isoenzymes designated as APase I, APase II and APase III in the order of their elution at 0.0 mM, 75 mM and 125 mM NaCl gradients, respectively. Each isoenzyme was further purified by gel filtration on Sephadex G-150 column with respective purification folds of 12.1, 10.6 and 9.9. APase I, APase II and APase III were found to be homogeneous as indicated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with apparent molecular weights of 79 Kda, 76 Kda and 66 Kda, respectively. All the three isoenzymes appear to possess only a single polypeptide chain each since both SDS-PAGE and electrophoresis in native gels (without SDS and β-mercaptoethanol) resulted in the appearance of single protein bands.

The purified PM-APases gave identical pH-activity profiles exhibiting pH-optimum at pH 5.0. The isoenzymes were found to exist in monomeric forms at pH 5.0 containing single polypeptide chains, but at pH 7.2 conversion of monomeric forms to higher dimeric forms was observed, with subsequent dissociation into monomers on lowering the pH to 5.0 from pH 7.2. The dimeric forms of PM-APases at pH 7.2 were found to consist of two polypeptide chains of identical molecular weight. The molecular weight of the dimeric forms of APase I, APase II and APase III were found to be 159 Kda, 150 Kda and 130 Kda, respectively. The formation of high molecular weight forms from monomeric forms *in vitro* was also confirmed by actually separating the two forms of each isoenzyme by Sephadex G-150 chromatography. Thus, *in vitro* association and dissociation of PM-APases appears to be a pH-dependent phenomenon and is suggested to be the mechanism by which the activity of the APases is regulated in plant

storage cells during germination when large pH variation is likely to occur. It is also suggested that APases may exist as dimers *in vivo*.

The substrate specificities of the PM-APases was also found to be pH-dependent with degree of specificity for each isoenzyme being significantly different from the other. At pH 7.2, the APase isoenzymes showed greater substrate specificity towards nucleotide phosphates and sugar phosphates, whereas, at pH 5.0 all the isoenzymes showed maximum specificity towards p-nitro phenyl phosphate (pNPP). The K_m and V_{max} values of APase I, APase II and APase III for the hydrolysis of pNPP as determined by Lineweaver-Burk plots were 0.43 mM, 0.50 mM , 0.48 mM and $2.1x10^2$ µM/min/mg, $2.8x10^2$ µM/min/mg and $2.4x10^2$ µM/min/mg, respectively. Orthophosphate (PO_4^{3-}) inhibited APase I (K_i = 1.42 mM) and APase II (K_i = 0.83 mM) competitively, but APase III (K_i = 0.48 mM) was inhibited noncompetitively. PM-APases also showed highly differential response towards various cations and anions thereby demonstrating that the three isoenzymes are different from each other and are not the modified or proteolytic products of the same enzyme.

Active sites of APase I, APase II and APase III were found to contain histidine residues as judged from their inhibition by Rose-bengal, a histidine specific dye. Cysteine residues also appeared to be involved with the active sites of APase I and APase II but not APase III. Considerable amounts of Mn and a little amount of Zn were found to be strongly associated with the purified PM-APases. The presence of Cu and Fe was not detected in any of the PM-APase isoenzymes. The presence of Mn in the PM-APases is especially interesting because all the other Mn-containing plant APases have been generally found to possess a characteristic violet colour and this, perhaps, is the first report of Mn-containing APases from a plant membrane without the characteristic violet colour.

APase I, APase II and APase III were found to be glycoproteins containing 50%, 27% and 30% carbohydrate, respectively. APase I was seen to contain D-man and GlcNAc while APase II and APase III were mainly found to contain D-man and D-glu as the main sugars. The nature of peptide-carbohydrate linkage appeared to be N-glycosidic in case of APase I but O-glycosidic in case of APase II and APase III. Sodium meta periodate treatment resulted in a sharp decrease in the activities of the APase isoenzymes suggesting a role for the carbohydrate moieties of APase I, APase II and APase III in maintaining the catalytic activity of the enzymes and keeping them in an active conformation.

Monensin was seen to bring about a decrease in the level of PM-APase with a concomitant increase in the level of GA-APase as compared to the control. Since monensin is thought to disrupt the traffic of proteins within plant cells, it seems that monensin blocks the intracellular transport of APase from GA to PM. It may, thus, be pertinent to suggest that APase present in peanut cotyledon cells must follow the route ER→GA→PM for intracellular transport and can be used as a model glycoprotein for studying the same in plant storage cells.

Gibberellic acid (GA_3) treatment was seen to bring about an accumulation of APase activity in PM but a decrease in APase activities in GA and ER fractions of peanut cotyledon cells unlike the situation observed in case of barley aleurones (93). It is suggested that if APases present in peanut cotyledon cells follow the route ER → GA → PM for intracellular transport and if GA_3-treatment brings about direct connections of ER with the PM as has been observed in case of GA_3 - treated barley aleurones (93), then higher activity of APase in PM fraction could be as a result of direct and faster transport

of APase from ER to PM with no intermediary passage through GA. Further work along these lines is in progress.

Antibodies raised against purified PM-APase isoenzymes were seen to immunoprecipitate APase activities from ER and GA fractions also, thereby, supporting our theory of transport of PM-APases via the route ER → GA → PM since it was reasoned that if PM-APases have to pass through ER and GA as well, antibodies raised against PM-APases should be able to immunoprecipitate APase activities from ER and GA also. It is further suggested that the carbohydrate moieties of APase I, APase II and APase III may act as antigenic determinants and that APases present in PM, GA and ER membranes may each contain a set of membrane-specific epitopes in their carbohydrate regions.

APase I, APase II and APase III were found to be immunologically related to each other and assumed to share some common epitopic regions. Anti-APase I immunoprecipitated 33.8% and 27.6% APase activity from APase II and APase III; anti-APase II precipitated 31% and 35.1% activities from APase I and APase III while anti-APase III immunoprecipitated 25% and 39% activities from APase I and APase II, respectively. The peanut cotyledon PM-APases were also found to be immunologically related to an APase isoenzyme (AP-I) of pea cotyledon PM and APase present in the epididymal extract of ram. It is suggested that APases from peanut cotyledon PM, pea cotyledon PM and ram epididymis have all evolved from a common ancestral gene and their common epitopic regions have remained conserved throughout the process of evolution.

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Solubilization, Purification and Characterization of an Acid Phosphatase from the Plasma Membranes of Peanut Cotyledons

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Three isoenzymes of acid phosphatase (EC 3.1.3.2) (APase I, APase II and APase III) from peanut cotyledon plasma membranes were purified 69, 26 and 16 fold by selective solubilization with octylglucoside in the presence of $MgCl_2$ and EDTA followed by ion-exchange chromatography on DEAE-cellulose. The APase I was found to be relatively pure by polyacrylamide gel electrophoresis giving only one prominent protein band with 0.23 electrophoretic mobility relative to bromophenol blue. The enzyme appeared to be a glycoprotein containing 50% carbohydrate. On SDS-polyacrylamide gel electrophoresis the enzyme protein band was dissociated into two bands corresponding to apparent molecular weights of 46.7 kDa and 50.1 kDa, indicating the presence of two subunits. The values of optimum *p*H, K_m and V_{max} of the enzyme were 4.7, 10 m*M* and 5.5 μmole/min/mg for *p*-nitrophenyl phosphate as substrate, respectively. The enzyme was non-competitively inhibited by inorganic phosphate (K_i = 34.5 m*M*). The purified enzyme was unstable and lost its total activity within 12 hr at 0°C. Thermodynamic parameters for the enzyme-*p*-nitrophenyl phosphate interaction were determined.

Acid phosphatases (EC 3.1.3.2, orthophosphoric monoester phosphohydrolases) are widely distributed in nature and have been identified in a large variety of organisms and tissues[1]. The enzymes are also ubiquitous in cell membranes and cell walls[2]. In yeast cells, the acid phosphatases reside on the outer side of the cell membrane and have been used as secretory markers[3-7]. Although soluble cytoplasmic acid phosphatases have been purified from several animal[2,8,9] and plant[10,11] sources, the membrane-bound enzymes, especially in plants, are poorly characterized and little is known about their functions. Perhaps one of the difficulties lies in purifying the enzymes due to their instability in the solubilized form. Recently, however, Crasnier *et al.*[12] have purified and characterized the acid phosphatase from the sycamore cell walls and it is expected that in due course of time more reports would appear on the purification and characterization of membrane-bound enzyme from different sources. In the present paper, we describe isolation and characterization of an acid phosphatase devoid of 5′-nucleotidase activity from the plasma membranes of peanut cotyledons.

Materials and Methods

p-Nitrophenyl phosphate and adenosine-5′-monophosphate used as substrates were purchased from Pierce Chemical Co. (USA). Tris, *n*-octyl-β-D-glucopyranoside, Triton X-100, sodium dodecyl sulphate (SDS), sodium deoxycholate, taurooleonyl cholate and Nonidet P-40 were obtained from Sigma. Acrylamide, N,N′-methylene-bisacrylamide, N,N,N′,N′-tetramethyl ethylenediamine and DEAE-cellulose (Servacel DEAE 23 SH) were obtained from Serva (FRG). The protein standards for molecular weight determination were obtained from Bio-Rad (USA). All the other chemicals used were reagent grade obtained from various commercial sources. Peanut seeds (large variety) were purchased from the local seed stores.

Preparation of membrane fraction — Unless stated otherwise, all operations were carried out at 0-4°C. Peanut seeds (*Arachis hypogea* L.) were germinated on four pieces of damp cheese-cloth placed one over the other in plastic trays kept in dark in a seed germinator for indicated periods at 100% humidity and 35°C. Approx. 200 cotyledons (80-100 g, fresh wt) of appropriate age were used for the preparation of microsomal and plasma membrane fractions, as described previously[13].

Enzyme assays — Acid phosphatase activity was measured by a slightly modified method of Odds and Hierholzer[14] using *p*-nitrophenyl phosphate as substrate. The incubation mixture (1 ml) contained 50-100 μg enzyme protein, 2.5 m*M* *p*-nitrophenyl phosphate and 50 m*M* acetate buffer (*p*H 5.0). The reaction was terminated after 15 min incubation at 30°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 enzyme had been added after terminating the reaction. Specific activity was expressed as μmole *p*-nitrophenol produced per minute per milligram protein. 5′ Nucleotidase activity was assayed as described previously[13].

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Solubilization and purification of plasma membrane acid phosphatase — All steps of solubilization and purification were performed at 0-4°C. The membrane fraction (25 mg protein) was suspended in 10 ml of 50 m*M* Tris-HCl (*p*H 7.2) containing 0.25 *M* sucrose, 0.04% (v/v) β-mercaptoethanol, 20 m*M* $MgCl_2$, 5 m*M* EDTA and 0.5% octylglucoside. The suspension was gently stirred on a Vortex mixer for 2 min followed by three strokes in Teflon homogenizer. The homogenate was then immediately centrifuged at 105,000 × *g* for 60 min. The supernatant containing the solubilized enzyme was used for further purification by DEAE-cellulose column as described below:

The solubilized enzyme was dialyzed overnight against 10 m*M* Tris-HCl buffer (*p*H 7.2). The dialyzed enzyme (*ca* 16.5 mg protein) was loaded on a DEAE-cellulose column (1.5 × 10 cm) pre-equilibrated with the dialyzing buffer. The column was washed with 20 ml of the equilibrating buffer to remove the unabsorbed proteins. The absorbed proteins were then eluted batch-wise using successively 20 ml of equilibrating buffer containing 50 m*M*, 100 m*M*, 200 m*M* and 300 m*M* NaCl. One ml fractions were collected at a flow rate of 0.2 ml/min and assayed for the activity of acid phosphatase, 5′-nucleotidase and protein.

Polyacrylamide gel electrophoresis — Polyacrylamide gel electrophoresis (PAGE) was carried out on 7.5 and 10% acrylamide gel in cylindrical tubes (0.5 × 9 cm) with and without SDS using the slightly modified procedures of Weber and Osborn[15] and Davis[16] as described previously[13]. Protein bands were located by staining with 0.5% Coomassie Brilliant Blue followed by destaining in 7% acetic acid. Staining for carbohydrates was done by the periodate-fuchin procedure[17]. The mobilities were determined relative to bromophenol dye and the protein standards used were: phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 68000); ovalbumin (M_r 43000); carbonic anhydrase (M_r 30000); and soybean trypsin inhibitor (M_r 21000).

For isolation, the PAGE was carried out at 4°C and from multiple gels, the glycoprotein band showing enzyme activity was cut, homogenized in 2 ml of 50 m*M* sodium acetate buffer (*p*H 5.0) containing 100 m*M* NaCl and centrifuged at 10,000 × *g* for 20 min. The supernatant containing APase I was concentrated by ultrafiltration.

Determination of kinetic and thermodynamic parameters — The apparent inhibition constant (K_i) value was obtained from the replot of the slopes of the Lineweaver-Burk plots *versus* inhibitor concentration[18]. Thermodynamic parameters, viz. energy of activation (E_{act}), enthalpy change (ΔH), free energy change (ΔG) and the entropy change (ΔS) were determined as described previously[19] using K_m and V_{max} values obtained at different temperatures.

Other methods — Total carbohydrate content was measured by the phenol/H_2SO_4 method[20] with glucose as standard. Protein was estimated by a modification[21] of the method of Lowry *et al.*[22] in the presence of 0.1% SDS using bovine serum albumin as standard. All samples were normalized with regard to detergent and buffer concentrations. β-Mercaptoethanol was removed by heating the protein sample at 60°C for 30-60 min in a water-bath before performing the protein assays.

Results

Membrane-bound acid phosphatase and 5′-nucleotidase activities in germinating peanut cotyledons — The variation in the activities of acid phosphatase and 5′-nucleotidase associated with the microsomal membrane fraction (14000-105000 × *g* pellet) of peanut cotyledons during 14 days of germination is shown in Fig. 1. Both enzymes exhibited maximum activitity between 6th and 7th day of germination at 35°C. However, the developmental patterns of the two enzymes were markedly different. For instance, 5′-nucleotidase emerged only after 48 hr of imbibition, the period by which the acid phosphatase had attained 40% of the maximal level. Similarly, while after 14 days germination, the 5′-nucleotidase activity was insignificant, that of acid phosphatase was substantially high. The specific activity was even higher. Thus, by using peanut cotyledons of appropriate age it was possible to prepare the desired

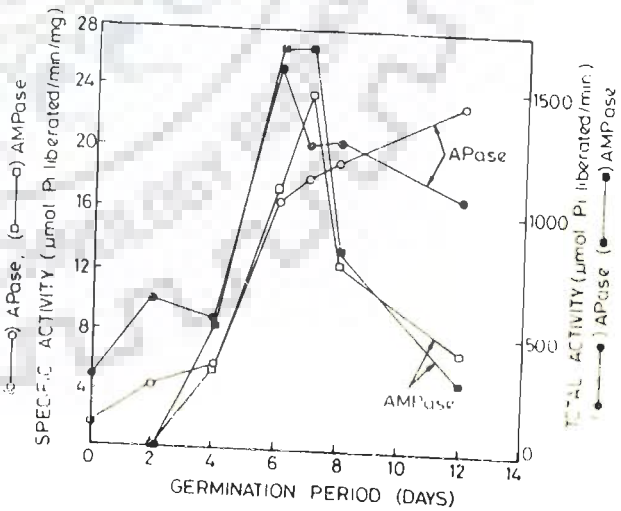

Fig. 1 — Effect of germination period on the levels of membrane-bound acid phosphatase and 5′-nucleotidase [Germination was carried out in dark at 35°C and 100% humidity. Microsomal membrane fraction (14000-105000 × *g* pellet) was used for the enzyme and protein assays as described under Materials and Methods]

membrane fractions devoid of 5′-nucleotidase activity for the isolation of acid phosphatase.

Purification of plasma membrane acid phosphatase — Table 1 summarizes the purification of the plasma membrane acid phosphatase from the peanut cotyledons. The use of a membrane fraction devoid of 5′-nucleotidase activity formed the crucial step in the purification of the enzyme, as it was difficult to remove the contamination of 5′-nucleotidase specially when it also exhibited the acid phosphatase activity[13] with similar solubilization characteristics.

Fractionation of plasma membranes from Golgi apparatus and endoplasmic reticulum by sucrose gradient resulted in 2.4-fold purification of acid phosphatase. The next step involved the use of a suitable detergent which would solubilize the membrane-bound enzyme in both active and stable form. The data given in Table 2 show that of the five detergents (deoxycholate, taurocholate, Triton X-100, Nonidet P-40 and octylglucoside) used, the octylglucoside was clearly the best one for the solubilization of acid phosphatase of plasma membrane. Under the conditions employed, nearly two-third of total enzyme activity was solubilized by octylglucoside and the specific activity increased about 6.7-fold. Triton X-100 and Nonidet P-40 were only half as effective. The ionic detergents, deoxycholate and taurocholate, were strongly inhibitory. Mg^{2+} were found to have a profound effect on the selective solubilization of the enzyme from the plasma membrane (Fig. 2). At the optimal concentration of Mg^{2+} (20 m*M*), there was 2.5- and 3-fold increase in specific and total activities of the solubilized enzyme, respectively.

Further purification of the solubilized enzyme was done on DEAE-cellulose column. The elution profiles of the acid phosphatase activity and proteins are presented in Fig. 3. A significant amount of acid phosphatase activity as well as of protein was eluted in the washings. Three acid phosphatase containing protein peaks (I, II and III) were obtained at 50 m*M*, 100 m*M* and 200 m*M* NaCl gradients respectively, indicating the presence of multiple forms of the enzyme in peanut cotyledon plasma membranes. The isoenzymes in peaks I, II and III were referred to as acid phosphatase (APase) I, APase II and APase III.

Top three fractions in each peak were pooled and the fold purification of enzyme I, II and III was 69.4, 26.4 and 16.1 with yields of 29.1, 8.8 and 5.3%, respectively. APase I was found sufficiently pure by

Table 1 — Purification of Peanut Cotyledon Plasma Membrane Acid Phosphatases

Fraction	Total protein (mg)	Total activity (μmole *p*-NP/min)	Sp. activity (μmole *p*-NP/min/mg protein)	Purification (fold)	Yield (%)
Crude microsomal membrane	331.0	1324.4	4.4	—	100.0
Plasma membrane	70.3	739.6	10.5	2.4	55.8
Octylglucoside extract	16.5	491.7	29.8	6.7	37.1
DEAE-cellulose					
Peak I	1.26	384.80	305.4	69.4	29.1
Peak II	0.68	79.22	116.5	26.47	8.8
Peak III	0.67	47.43	70.8	16.1	5.3

Table 2 — Solubilization of Membrane-bound Acid Phosphatase with Different Detergents

[Solubilization of the membrane-bound enzyme with different detergents was performed under identical conditions as described in Materials and Methods]

Fraction	Total protein (mg)	Total activity (μmole *p*-NP/min)	Sp. activity (μmole *p*-NP/min/mg protein)	Activity solubilized (%)	Protein solubilized (%)
Plasma membrane	10.75	112.9	10.5	—	—
Detergent extract[a]					
Deoxycholate	0.94	4.7	2.21	4.2	8.7
Taurocholate	0.89	6.1	3.09	5.4	8.3
Triton X-100	1.01	33.1	14.96	30.0	9.4
Nonidet P-40	0.99	33.2	15.00	29.4	9.2
Octylglucoside	1.05	73.9	30.30	65.5	9.8

[a](105000 × *g* supernatant).

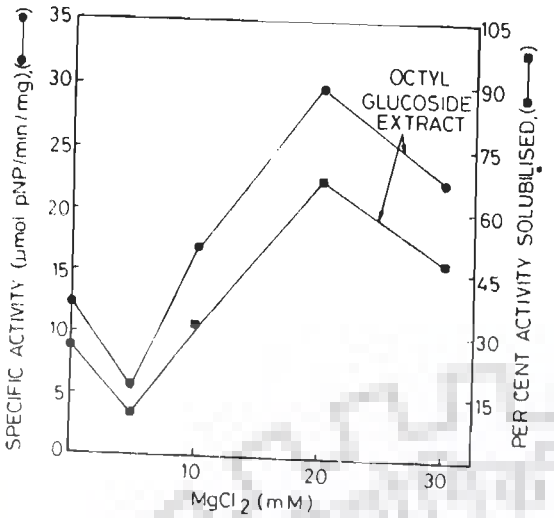

Fig. 2 — Effect of Mg^{2+} concentration on the solubilization of membrane-bound acid phosphatase by octylglucoside. [Solubilization was carried out as described under Materials and Methods]

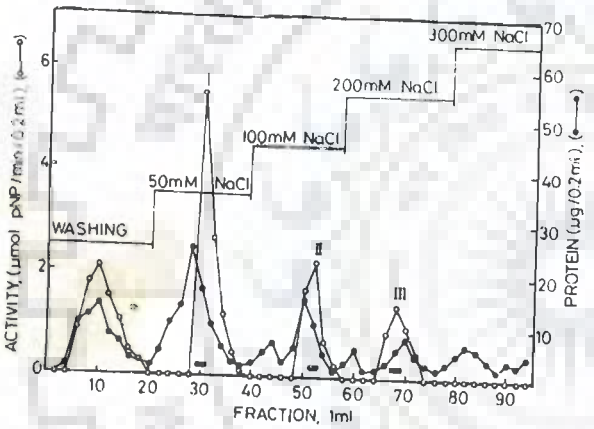

Fig. 3 — DEAE-Cellulose chromatography of the dialyzed octylglucoside solubilized acid phosphatase [A 10 ml sample (16.5 mg protein) was applied to the column (1.5 × 10 cm) and the absorbed proteins were eluted by step-wise gradient of NaCl as described in the text. Fractions indicating by bar were pooled and used for further studies]

polyacrylamide gel electrophoresis with only one major protein band with enzyme activity corresponding to an electrophoretic mobility of 0.23, relative to bromophenol blue. Two faint protein bands of electrophoretic mobility of 0.17 and 0.5 were also observed. The major protein band, whose intensity increased during purification, also gave positive staining for carbohydrates with periodate-Schiff's reagent[17], indicating the glycoprotein nature of the acid phosphatase I. On SDS-PAGE, the major protein band showing enzyme activity was dissociated into two bands; a major and a minor corresponding to molecular weights of 46.7 kDA and 50.1 kDA respectively, suggesting the presence of two subunits in the molecule of the enzyme. Since PAGE resulted in sufficient loss of enzyme activity, this step was not used on preparative scale. The APase I from DEAE-cellulose was used for characterization.

Properties of the plasma membrane APase I—The partially purified APase I showed optimum *p*H, K_m and V_{max} for the *p*-nitrophenyl phosphate of 4.7, 10 m*M* and 5.5 μmole/min/mg, respectively. The values of E_{act}, ΔH, ΔG and ΔS for the formation of enzyme-substrate (*p*-nitrophenyl phosphate) complex were found to be 6155 cal/mole, 4.25 kcal/mole, 2.77 kcal/mole and 4.9 cal/deg/mole, respectively. The positive ΔH indicates that the reaction between APase I and *p*-nitrophenyl phosphate is endothermic. Similarly, the positive ΔG is also indicative of the endergonic reaction. The relatively low positive ΔS suggests that unfolding of the polypeptide chain into a less ordered system has not occurred.

APase I was inhibited by KH_2PO_4 in a non-competitive manner with an apparent K_i of 34.5 m*M* and it neither showed any hydrolytic activity towards 5′-mono-, di- and tri-nucleotides nor was inhibited by these compounds. The purified enzyme was highly unstable and lost its total activity within 12 hr at 0° or − 20°C.

Discussion

The purpose of this study was to characterize the plasma membrane acid phosphatase of peanut cotyledons. One of the major difficulties was the separation of 5′-nucleotidase from the acid phosphatase as the two enzymes have similar solubilizing characteristics[13] and in general acid phosphatases exhibit 5′-nucleotidase activity[1]. Further, even the apparently pure 5′-nucleotidase preparation (homogeneous by SDS-PAGE) was found to show acid phosphatase activity as well[13,24]. The results described here show that by choosing the germinating cotyledons of proper age interference of 5′-nucleotidase in the purification of plasma membrane acid phosphatase was avoided. In addition, the results show that the plasma membrane acid phosphatase was devoid of 5′-nucleotidase activity which may be indicative of the organelle specificity.

The treatment of plasma membrane fraction with octylglucoside in the presence of Mg^{2+} and EDTA at a protein to detergent ratio of 1:2 resulted in a selective and true solubilization[23] of the membrane-bound enzyme, since the enzyme remained soluble, stable and active even after the removal of detergent.

The results of anion-exchange chromatography have revealed the presence of multiple ionic species of acid phosphatase in the peanut plasma membranes. It was not unexpected, however, as plant seeds have been shown to contain isoenzymes of acid phosphatase[10,11]. The partially purified enzyme

obtained from the DEAE-cellulose column was very unstable losing its total activity within 12 hr at −20°C. Thus, further purification could not be achieved. It appears likely that some stabilizing factors like phospholipids or lectins were lost during purification on ion-exchange column. Recently, Ferens and Morawiecka[11] have suggested that lectins provide a protective action to acid phosphatases.

The peanut cotyledon plasma membrane acid phosphatase appears to be a glycoprotein. In this respect it confirms the general nature of acid phosphatases[2-4,10,11,14]. It is now well established that the proteins which are destined for secretion or to become the part of the plasma membrane are synthesized in rough endoplasmic reticulum (RER), vectorially discharged into the lumen of RER, translocated to Golgi vesicles and finally packed in secretory vesicles for transport to plasma membranes[25]. Thus, the plasma membrane acid phosphatase in peanut cotyledons should also follow the above mentioned path as was shown in the case of yeast[4]. It is suggested that acid phosphatase may serve as a model glycoprotein for studying the intracellular transport of proteins in plant storage cells.

Acknowledgement

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PURIFICATION AND CHARACTERIZATION OF 5′-NUCLEOTIDASE FROM THE GOLGI APPARATUS OF PEANUT COTYLEDONS

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An adenosine 5′-monophosphatase (AMPase) has been purified from the Golgi-apparatus (GA) of peanut cotyledons by first selectively solubilizing the enzyme with 0.5% non-ionic detergent, *n*-octyl-β-D-glucopyranoside, at a protein-to-detergent ratio of ?:3 in the presence of 20 mM $MgCl_2$ and 5 mM EDTA, and then by ion-exchange chromatography on DEAE-cellulose. The GA-AMPase is a glycoprotein having 38.5% carbohydrate and apparent M_r = 53.7 kDa. It was inhibited competitively by ADP (K_i = 3.4 mM) and non-competitively by NaF (K_i = 41 mM). The values of optimum pH, K_m and V_{max} for the hydrolysis of AMP were 5.0–5.5, 0.9 mM and 0.4 mM/min per mg, respectively. The enzyme is highly specific for AMP with no hydrolysis of ATP, ADP, GTP, GDP, GMP, UTP, UDP, UMP, CTP and CMP. The purified AMPase from GA resembled the plasma membrane (PM) enzyme closely, except for a slightly lower carbohydrate content. It is suggested that GA-AMPase may be a precursor of the PM-AMPase in peanut cotyledon cells.

Key words: Arachis hypogaea; AMPase; 5′-nucleotidase; Golgi apparatus; purification

Introduction

In a previous communication from this laboratory we reported the purification and properties of a glycoprotein AMPase from the plasma membrane (PM) of peanut cotyledons [1]. During the course of these studies it was observed that besides the PM, a sizeable amount of AMPase activity was also associated with the membrane fraction containing mostly Golgi-apparatus (GA). Since the GA is involved in the biosynthesis, modification and intracellular transport of macromolecules in both plant and animal cells and it receives the biosynthetic products from the endoplasmic reticulum (ER) which are destined for secretion or to become the part of the PM [2–5], the AMPase found in GA may be the precursor (or same) of the enzyme present in the PM. Working on this hypothesis we prepared highly enriched fraction of GA, which was essentially free from PM and ER, purified AMPase and compared its properties with that of the PM enzyme. Both GA- and PM-AMPases show similar properties with slight differences.

Materials and methods

Materials

Adenosine 5′-monophosphate (AMP) and other 5′-ribonucleotides, glucose-6-phosphate, glucose-1-phosphate, *p*-nitrophenyl phosphate, used as substrates, were purchased from Pierce Chemical Co. (U.S.A.). Tris, *n*-octyl-β-D-glucopyranoside, Triton *X*-100, sodium dodecyl sulfate (SDS), sodium deoxycholate, taurooleonyl cholate, Nonidet NP-40 and UDP-glucose were obtained from Sigma. Acrylamide, *N,N′*-methylene-bisacrylamide *N,N,N′,N′*-tetramethyl ethylenediamine, and DEAE cellulose (Servacel DEAE 23 SH) were obtained from Serva (F.R.G.). The protein standards for molecular weight determination were obtained from Bio-Rad (U.S.A.). Radioactive UDP-[^{14}C]

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glucose (260 Ci/mol) and GDP-[^{14}]mannose (228 Ci/mol) were purchased from the Radiochemical Center, Amersham (U.K.). All other chemicals were reagent grade from various commercial sources. Peanut seeds (large variety) were purchased from the local seed stores.

Preparation of GA membrane fraction

The crude microsomal membrane fraction (12 000–105 000 × *g* pellet) containing PM, GA and ER was prepared from 7-day-old germinating peanut cotyledons as described previously [1]. The plasma membrane fraction was separated from the rest by a step gradient of 34/20% (w/w) sucrose as described by DuPont et al. [6]. The membrane fraction staying at 34/20% sucrose gradient interface was removed carefully with the help of a Pasteur pipette and fractionated further into the GA and microsomes by the sucrose density gradient method described by Green [7] with slight modifications. The 34/20% sucrose gradient interface membrane fraction was diluted with an equal volume of homogenizing buffer and pelleted by centrifugation at 105 000 × *g* for 60 min. The pellet was suspended in 4 ml of 25 mM Tris–HCl (pH 7.2) containing 20% sucrose and 1 mM β-mercaptoethanol. This was then floated carefully on a sucrose gradient system prepared by layering 7 ml of each of 43%, 37% and 25% sucrose solution successively in the same buffer. The system was immediately centrifuged at 105 000 × *g* for 3 h. A major band was obtained at the 37/25% sucrose gradient interface. The supernatant part was removed carefully without disturbing the interface and saved for the preparation of microsomes. The membrane fraction at the interface was removed carefully as before. This fraction represented the GA as the activity of the marker enzyme, glucan synthase I, was exclusively localized in this fraction and no activity of glucan synthase II, a marker enzyme for the PM, was found in it.

The glucan synthase I activity was measured as described by Green [7] using 100 μg membrane protein, 100 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 10 mM cellobiose, 4 mM EDTA, 2 mM β-mercaptoethanol and 0.1 μCi UDP-[^{14}C]glucose (6 nmol) in a total volume of 0.1 ml. The glucan synthase II activity was determined as described previously [1]. The reaction mixture contained the following in a total volume of 0.1 ml:100 μg membrane protein, 50 mM Tris-HCl (pH 7.5), 0.1 μCi UDP-[^{14}C]glucose (260 Ci/mol) and 0.5 mM unlabeled UDP-glucose. The mannosyl transferase, used as a marker enzyme for ER [8], was measured by the method of Lehle et al. [9] using dolichol monophosphate as the acceptor substrate and GDP-[^{14}C]mannose as donor of mannosyl group.

Enzyme assays

5′-Nucleotidase activity was measured with 5′-AMP as substrate as described by Riemer and Widnell [10] with slight modifications. The standard reaction mixture, unless stated otherwise, contained 1 – 2.5 mM AMP, 50 mM sodium acetate buffer (pH 5.0) and 0.1 ml of the enzyme preparation (8–70 μg protein, depending upon the form of enzyme assayed) which was added last, in a total volume of 1.0 ml; control incubations contained no substrate. Incubation was carried out at 30°C for 15 min and the enzyme activity was terminated by adding 0.5 ml cold 20% trichloroacetic acid. Protein was removed by centrifugation and P_i determined in the supernatant fluid by the procedure of Fiske and SubbaRow [11]. Under the assay conditions, P_i release was linear with time up to 60 min and enzyme concentration provided not more than half of the substrate was hydrolyzed at the longest time interval (data not shown). The specific activity of AMPase corresponds to the dephosphorylation of 1 μmol of 5′-AMP/min per mg protein. When detergents especially Triton *X*-100 and Nonidet NP-40 were present in the assay system, the P_i was determined by the modified Fiske and SubbaRow method as described by Dulley [12]. In this method P_i analyses were done in the presence of 3% SDS which eliminated interference due to Triton *X*-100 and other detergents.

Solubilization and purification of AMPase

All steps of solubilization and purification were carried out at 0–4°C. The membrane fraction obtained above was suspended (30 mg/ml)

in 50 mM Tris–HCl buffer (pH 7.2) containing 0.25 M sucrose, 0.04% (v/v) β-mercaptoethanol, 20 mM $MgCl_2$ and 5 mM EDTA with a Teflon tissue homogenizer. To 1 ml suspension (30 mg protein) 11 ml of 0.6% (w/v) *n*-octyl-β-D-glucoside in the same buffer was added dropwise with stirring so that the final detergent concentration and the protein-to-detergent ratio were 0.5% and 2/3, respectively. The enzyme was solubilized by gentle agitation on a Vortex mixer for 2 min followed by three strokes in a Teflon homogenizer and then immediately centrifuged at 105 000 × *g* for 60 min to separate the soluble enzyme from the insoluble material. The supernatant containing AMPase was dialyzed 6 h against 100 vol. of 10 mM Tris–HCl buffer (pH 7.2). The dialyzed enzyme (11 ml, 4 mg protein) was applied on a DEAE-cellulose column (1.5 × 10 cm) which was previously equilibrated with the same buffer. The column was washed with 20 ml buffer to remove unabsorbed proteins. The absorbed proteins were eluted by a linear gradient from 0 to 300 mM NaCl using a single mixing container with 50 ml of 10 mM Tris–HCl buffer (pH 7.2) and reservoir with 50 ml of the same buffer containing 300 mM NaCl. The flow rate was 0.2 ml/min and 1-ml fractions were collected. Aliquots (0.2 ml) from every other fraction were analyzed for the protein content and AMPase activity. The peak enzyme containing fractions were pooled, concentrated by ultrafiltration and analyzed by polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate (SDS). In some cases unconcentrated fractions were used for various assays.

SDS-Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out on a 10% gel in cylindrical tubes (0.5 × 9 cm) in the presence of 0.1% SDS according to the procedure described by Weber and Osborn [13]. Proteins were first heated for 5 min at 100°C in 0.01 M phosphate buffer (pH 7.4), containing 1% SDS and 1% β-mercaptoethanol. Reference proteins used for molecular weight determination were also subjected to the same treatment. Protein samples (40–100 μg) were layered through the electrode buffer in 40% sucrose solution. Bromophenol blue was used as tracker dye. The gels were run at a constant current of 5 mA/tube for 2.5 h at room temperature. Protein bands were located by staining with 0.5% Coomassie Brilliant blue in water/methanol/acetic acid solution (50:45:7, by vol.) for 6–8 h and were destained with 7% acetic acid. The gels were stored in 7% acetic acid. The mobilities were determined relative to bromophenol dye in 7% polyacrylamide gels without SDS at room temperature at pH 7.4 in 0.01 M phosphate buffer. The molecular weight standards were phosphorylase *b*, M_r = 94 kDa; bovine serum albumin, M_r = 68 kDa; ovalbumin, M_r = 43 kDa; carbonic anhydrase, M_r = 30 kDa; soybean trypsin inhibitor, M_r = 21 kDa and lysozyme, M_r = 14.3 kDa. The molecular weight of the purified AMPase was computed from the calibration plot of $\log_{10} M_r$ vs. relative mobility of standard proteins. Molecular weight results were based on an average of three independent electrophoretic runs.

Kinetic studies

K_m and V_{max} values were obtained from the Lineweaver-Burk plots using AMP and *p*-nitrophenyl phosphate (*p*-NPP) as substrates in 50 mM sodium acetate buffer (pH 5.0). Enzyme assays with at least five substrate concentrations (AMP, 0.25–5 mM, and *p*-NPP, 15–50 mM) were carried out for 10 min with 10 μg enzyme. Values of apparent inhibition constant (K_i) were computed from the replots of slopes of a set of four Lineweaver-Burk plots, obtained in the presence of a fixed inhibitor concentration (NaF,0,1,10,20 and 30 mM; or ADP, 0,1,2.5 and 3.5 mM), vs. inhibitor concentration. The intercept on *X*-axis equals the value of $-K_i$.

Other methods

Total carbohydrate content was measured by the phenol/H_2SO_4 method [14] with glucose as standard. Protein was estimated by the method of Lowry et al. [15] as modified by Peterson [16] in the presence of 0.1% SDS using bovine serum albumin as standard. All samples were normalized with regard to detergent and buffer concentrations. β-Mercaptoethanol was removed by heating the protein sample at 60°C for 30–60 min in a water bath before performing

Table I. Distribution of marker enzymes and AMPase in various membrane fractions of peanut cotyledons. Peanut cotyledons (200) were used for preparing various membrane fractions. Glucan synthase I (GS-I), glucan synthase II (GS-II) and mannosyl transferase (MTase) were used as marker enzymes for Golgi apparatus, plasma membrane (PM) and smooth endoplasmic reticulum (SER) fractions, respectively. The enzymes were assayed as described under Materials and methods.

Fraction	Activity of marker enzymes (radioactivity incorporated)			AMPase activity		
	GS-I (cpm/mg)	GS-II (cpm/mg)	MTase[a] (cpm/mg)	Total activity (μmol P_i min)	Spec. act.- (μmol P_i min/mg)	Percent of total activity
Crude membranes (12 000–105 000 × *g* pellet)	3314	8100	61 288 (21 000)	2399.4	26.6	100
Plasma membranes	1230	88 246	3080 (1683)	1400.0	86.0	58.3
Golgi apparatus	17 856	1625	18 661 (7208)	565.7	37.9	23.5
Smooth endoplasmic reticulum	1972	3224	53 3120 (38 199)	298.3	26.8	12.4

[a]Values in parentheses were obtained without dolicholmonophosphate as exogeneous lipid acceptor of mannose from GPD-[^{14}C]mannose as donor substrate.

the protein assay according to the method of Lowry et al.

Results

Preparation of Golgi apparatus membrane fraction and distribution of AMPase among various membrane fractions of peanut cotyledons.

The total crude microsomal fraction (12 000–105 000 × *g* pellet) from 7-day-old germinating cotyledons, containing mainly PM, GA and ER + ribosomes, was used as the starting material for the separation of various membrane fractions using step sucrose gradient centrifugation and specific marker enzymes. The results are shown in Table I. As judged from the distribution of various marker enzymes, specific for particular membrane fractions, the PM, GA and ER fractions were enriched by about 11-, 5.4- and 6.2-folds, respectively, relative to the crude membrane fraction and appeared to be nearly free from the cross contamination.

The distribution pattern of AMPase in various membrane fractions (Table I) shows that in the germinating cotyledons of peanut, approximately 58 and 23.7% of the total precipitable AMPase activity were associated with PM and GA fractions, respectively. The ER (Microsomes) which stayed in 25% sucrose supernatant phase accounted for only 12.4% of the total precipitable AMPase activity. In terms of specific activity, PM showed the highest specific activity (86 μmol/min per mg) followed by GA (37.9 μmol/min per mg) and ER (26.9 μmol/min per mg).

Purification of AMPase from GA

Table II summarizes the purification of AMPase from GA. The separation of GA from the crude membrane fraction (12 000–105 000 × *g* pellet) resulted in 1.24-fold purification relative to the crude fraction, with a yield of 23.7%. The solubilization of the membrane-bound enzyme by *n*-octylglucoside extract further increased the purification 3-fold, the yield at this stage was 7.7% with respect to the crude membrane fraction. However, if GA is taken as the starting material, yield would be about 32.5%. The value closely resembles the solubilization data of the PM enzyme by *n*-octylglucoside [1].

The final purification step was the ion

Table II. Purification of AMPase from the peanut cotyledon Golgi-membrane fraction. Golgi-apparatus membranes were obtained from the crude particulate fraction (12 000–105 000 × *g*) as described under Materials and methods. The purified Golgi-membranes (3.33 mg protein/ml) was extracted with 0.5% octylglucoside in presence of 20 mM $MgCl_2$ and 5 mM EDTA, followed by 105 000 × *g* centrifugation. The supernatant fraction was used as the solubilized form of enzyme. DEAE-cellulose peak I and II represent the pool of only top three fractions of each peak. The purification and specific activity data were computed relative to the crude membrane fraction.

Fraction	Total protein (mg)	Total AMPase activity (μmol P_i/min)	AMPase specific activity (μmol P_i/min per mg protein)	Purification (fold)	Yield (%)
Crude membrane fraction (12 000–105 000 × *g* pellet)	127.1	3128.5	24.6	–	100.00
Golgi-fraction (37/25% sucrose interface)	24.3	740.4	30.47	1.24	23.70
Octylglucoside extract (105 000 × *g* supernatant)	3.2	240.6	74.5	3.02	7.70
DEAE-cellulose					
Peak I	0.271	45.49	167.74	6.81	1.4
Peak II	0.232	27.68	119.35	4.85	0.9

exchange chromatography on DEAE-cellulose. The elution profiles of the AMPase activity and the protein are shown in Fig. 1. Two major peaks containing AMPase were eluted at 55 mM and 107 mM NaCl gradient. Of these, peak I accounted for most of the enzyme activity eluted from the column. The purification folds of AMPase in peaks I and II were 6.8 and 4.8 with per cent yields of 1.4 and 0.9, respectively. The actual purification fold and per cent yield will be more than the observed values, but for the presence of other non-specific phosphorylases in the crude as well as in the GA membrane fractions.

Homogeneity

On SDS-PAGE in the presence of 2-mercaptoethanol, the purified enzyme in DEAE-cellulose peak I gave a single protein band corresponding to an apparent M_r = 53.7 kDa (Fig. 2). The relative intensity of this band increased progessively during the successive steps of AMPase purification, indicating that the protein band was due to the enzyme. Since, electrophoresis was performed under completely dissociated conditions, the peanut cotyledon GA-AMPase seems to have only a single polypeptide chain. However, the presence of more than one polypetide chain of exactly the same size cannot be ruled out.

Substrate specificity

The substrate specificity of the purified GA-AMPase was tested using AMP, GMP, CMP, UMP, ADP, GDP, UDP, ATP, CTP, UTP, GTP, glucose-6-phosphate, glucose 1-phosphate and *p*-nitrophenyl phosphate as substrates at 2.5 mM concentration under standard assay conditions. The purified 5′-nucleotidase was specific for AMP (167 μmol P_i/min per mg) with no hydrolysis of other nucleoside phosphates, including ADP and ATP, and phosphorylated sugars. The *p*-nitrophenyl phosphate was hydrolyzed only at a one-fifth rate (33.5 μmol P_i/min per mg) of that of the AMP, indicating that *p*-nitrophenyl was a very poor substrate for the GA-AMPase.

Optimum pH and kinetic properties of the purified GA-AMPase

The purified GA-AMPase had a pH-optimum range of pH 5.0–5.5 for the hydrolysis of AMP. The enzyme activity declined rapidly below pH 5.0 and above pH 6.0. The K_m and V_{max} values

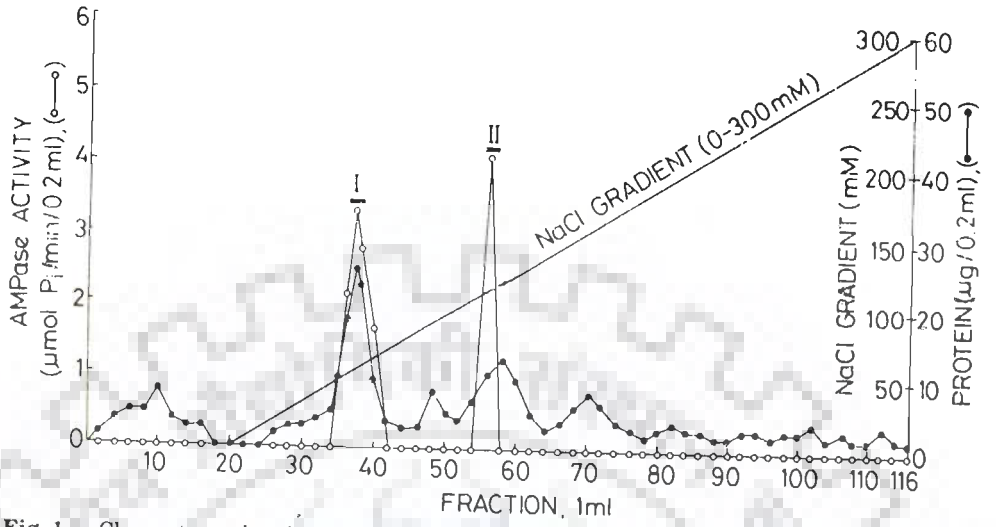

Fig. 1. Chromatography of the dialyzed octylglucoside solubilized AMPase (105 000 × *g* supernatant) on DEAE-cellulose column. A 9-ml sample (4.16 mg protein) was loaded on the column (1.5 × 10 cm), and the absorbed proteins were then eluted by a linear gradient of sodium chloride (0–300 mM) in 10 mM Tris–HCl (pH 7.2) as described in the text. The fractions indicated by the bar(-) were pooled and used for further studies. Fraction I (35–37) was found pure by SDS polyacrylamide gel electrophoresis.

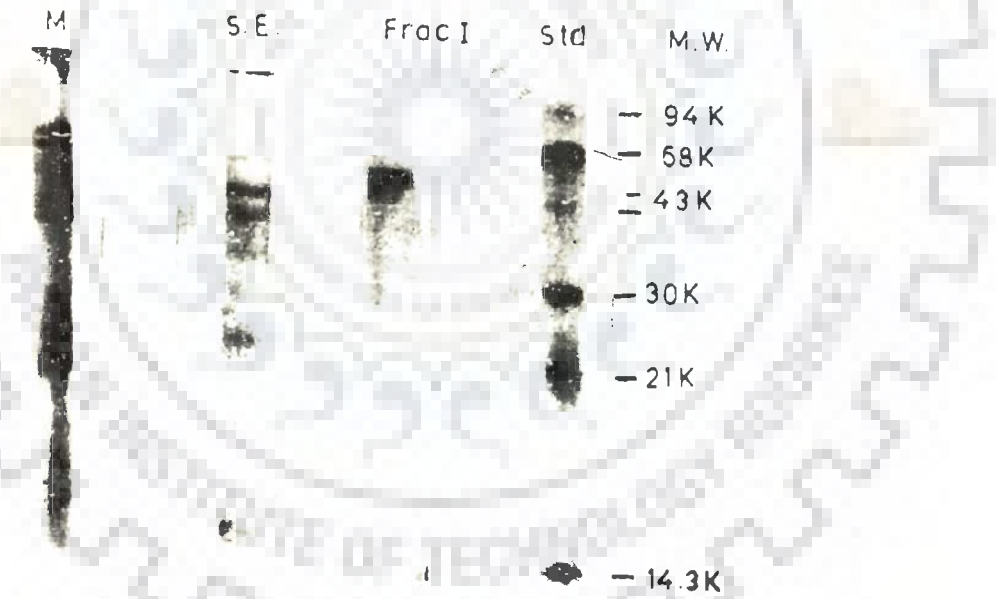

Fig. 2. SDS-polyacrylamide gel electrophoresis of the peanut cotyledon Golgi apparatus AMPase. Samples were prepared and handled as described under Materials and methods. Samples and amounts of protein loaded were: M, Golgi apparatus membrane fraction, 100 μg protein; S.E., octylglucoside solubilized enzyme (dialyzed 105 000 × *g* supernatant), 80 μg protein; Frac. I, DEAE-cellulose fraction I containing AMPase, 40 μg protein; and Std., molecular weight standard proteins (phosphorylase b, M_r = 94 kDa; bovine serum albumin, M_r = 68 kDa; Ovalbumin, M_r = 43 kDa; carbonic anhydrase, M_r = 30 kDa; soybean trypsin inhibitor, M_r = 21 kDa, and lysozyme, M_r = 14.3 kDa; 5 μg each). Standard proteins used were treated in the same manner as other protein samples were done. Electrophoresis was performed on 10% polyacrylamide gel under fully dissociating conditions. Mobilities were determined relative to the migration of bromophenol blue tracking dye. The molecular weight of the protein band in fraction I representing purified AMPase, as computed from the calibration plot of $\log_{10} M_r$ v. relative mobility of standard proteins, was found to be 53.7 kDa.

for AMP, as determined by Lineweaver-Burk plot (Fig. 3), were found to be 0.91 mM and 0.4 mM/min per mg, respectively. ADP and NaF inhibited the enzyme in a competitive and non-competitive manner (Fig. 3) with K_i values of 3.4 mM and 41 mM, respectively.

Effect of metal ions

The activity of GA-AMPase was not affected by Mg^{2+}, Ca^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, K^+, Li^+ and Na^+. Since Hg^{2+} ions do not produce any inhibitory effect on the enzyme, the SH-groups of cysteine residues are probably not involved in the interaction between the enzyme and the substrate.

Stability of GA-AMPase

The purified GA-AMPase was rendered totally inactive within 24 h at −20°C or at 0°C. On the other hand both membrane-bound as well as unpurified octylglucoside solubilized enzyme were stable for approximately 2 weeks at −20°C without any significant loss of activity indicating that some stabilizing factors, probably phospholipids, that were responsible for the stability of the enzyme in the crude form, were lost during purification on DEAE-cellulose.

Glycoprotein nature of GA-AMPase

The peanut cotyledon GA-AMPase was found to contain 38.5% (w/w) carbohydrate and like other plant 5′-nucleotidases [1,17] appears to be a glycoprotein.

Comparison of the properties of GA- and PM-AMPases

Table III shows the comparison between the properties of purified AMPase from GA and PM fractions of peanut cotyledons. The values of the optimum pH, K_m, electrophoretic mobilities, nature of inhibition by ADP and NaF including K_i values and elution behaviour from the

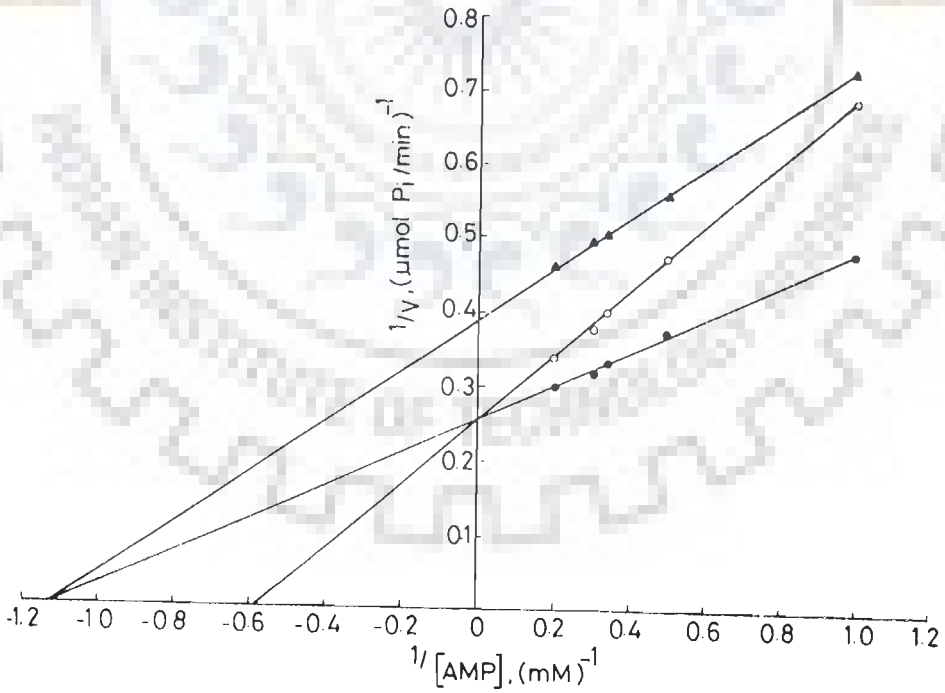

Fig. 3. Lineweaver-Burk plots of AMP hydrolysis by GA-AMPase. Standard assays with varying amounts of AMP were carried out for 10 min with 10 µg enzyme. Velocities are expressed as µmol/min per 10 µg of enzyme. ●, without ADP and NaF; ○, in presence of 2.5 mM ADP; ▲, 30 mM NaF.

Table III. A comparison of properties of AMPase from the Golgi and Plasma membrane fractions of peanut cotyledons.

Properties	Golgi-apparatus AMPase	Plasma membrane[a] AMPase
Molecular weight	53.7 kDa	55 kDa
Subunits present	Single polypeptide	Single polypeptide
Carbohydrate content	38.5%	42.7%
Electrophoretic mobility[b]	0.18	0.18
pH-optimum	5.0–5.5	5.0–6.0
K_m	0.91 mM AMP	1.08 mM AMP
V_{max}	0.4 mM/min per mg	0.8 mM/min per mg
Inhibition by ADP	Competitive (K_i = 3.4 mM)	Competitive (K_i = 3.4 mM)
Inhibition by NaF	Non-competitive (K_i = 41 mM)	Non-competitive (K_i = 35 mM)
Metal ion requirement[c]	None	None
Substrate specificity	Highly specific for AMP, no hydrolysis of other nucleotides	Highly specific for AMP, no hydrolysis of other nucleotides
Stability at −20°C	Total loss of activity within 24 h	Total loss of activity within 24 h
Elution from DEAE-cellulose column Peak I	At 55 mM NaCl gradient	At 50 mM NaCl gradient

[a]Data from Ref. 1.
[b]Electrophoretic mobility was determined in 7% polyacrylamide gel at 25°C and pH 7.4 in 0.01 M phosphate buffer, relative to bromophenol blue.
[c]Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, Ni^{2+}, K^+, and Li^+.

DEAE-cellulose column for both GA- and PM-AMPase were comparable. The only significant difference was found in the carbohydrate content of the two enzymes, which was somewhat lower in the case of GA (38.5%) than that of the PM-AMPase. The close resemblance in the properties of GA- and PM-AMPase suggests that the GA-AMPase may be precursor of the PM enzyme.

Discussion

The GA and the PM fractions used in this study were essentially free from cross contamination as judged by the absence of the marker enzymes of the PM (glucan synthase II) and the GA (glucan synthase I) from the GA and PM fractions, respectively. However, some contamination of ER in GA cannot be ruled out completely, since relatively small activity of the dolichol monophosphate – GDP – mannose: mannosyl transferase was found in this fraction (Table I).

The conditions of solubilization and scheme of purification of the GA-AMPase were exactly those used for the PM enzyme [1]. Thus, it was reasonable to presume that the AMPases obtained separately from GA and PM were representatives of the source from which the enzyme was isolated. As expected, the patterns of solubilization and elution from DEAE-cellulose of both GA- and PM-AMPase were the same except that a third peak (peak III), which eluted at 155 mM NaCl gradient from the PM [1] was not present in GA. In addition, the kinetic properties, response to metal ions (Mn^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Ni^{2+}, Hg^{2+}, K^+ and Li^+) and inhibitors (ADP and NaF), optimum pH, elec-

trophoretic mobility, stability, molecular weight and sub-unit structure of both GA and PM-AMPases were comparable. The only difference was found in the carbohydrate content. However, the difference in carbohydrate content, although small, was significant. This was not unusual as processing of carbohydrate moiety of glycoprotein also takes place in GA [2]. These results were interpreted to mean, but by no means prove, that the AMPase present in the GA may be the precursor (or same) enzyme, with slightly modified carbohydrate moiety, as found in the PM. In view of the above, it is suggested that AMPase may provide a model glycoprotein for studying the intracellular transport of proteins in plant storage cells.

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