STUDIES ON GLYCOPROTEIN ENZYMES OF PEANUT COTYLEDON CELL MEMBRANES : PURIFICATION TO HOMOGENEITY AND CHARACTERIZATION OF SOME GLYCOPOLYPEPTIDES WITH HIGHLY SPECIFIC AMPase ACTIVITY

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By

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Gratis

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled, "STUDIES ON GLYCOPROTEIN ENZYMES OF PEANUT COTYLEDON CELL MEMBRANES : PURIFICATION TO HOMOGENEITY AND CHARACTERIZATION OF SOME GLYCOPOLYPEPTIDES WITH HIGHLY SPECIFIC AMPase ACTIVITY", in fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY** of the University is an authentic record of my own work carried out during a period from **JULY, 1991 to JUNE, 1995** under the supervision of **Prof. (Dr.) C. B. SHARMA and Dr. RITU BARTHWAL.**

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

Signature of the Candidate

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

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Abstract

A plasma membrane localized low molecular mass (26.3 Kd) adenosine 5'-monophosphatase (PM-AMPase) and a Golgi apparatus localized high molecular mass (52.4 Kd) adenosine 5'-monophosphatase (GA- AMPase), from cotyledons of 7 -days old germinating peanut seedlings, have been purified to apparent homogeneity. This is evident by SDS-PAGE and Western blotting. Purification was carried out by selective solubilization of the membrane-bound enzyme with 1.0% CHAPS, at a protein-to-detergent ratio of 2:3 in the presence of Mg^{2+} ions and EDTA, followed by gel filtration on Sephadex G-150 and ion-exchange chromatography on DEAE-cellulose. Both PM-AMPase and the GA-AMPase are glycoproteins with 37% and 39% of carbohydrate content, respectively and have optimum pH in the range of 5.0 to 5.5. The K_m and V_{max} values for the PM-AMPase are 0.58 mM and 5.9 μ mole Pi/min/mg-protein and that for the GA-AMPase are 1.0 mM and 6.8 μ mole Pi/min/mg-protein, respectively. The PM-AMPase is highly specific for 5'-AMP. Other nucleotides, phosphorylated sugars and p-nitrophenyl phosphate (p-NPP) were not hydrolyzed. The purified GA-AMPase shows much broader substrate specificity and also catalyzes the hydrolysis of 5'-GMP, 5'-UMP and p-NPP at significant rates.

The purified enzymes are highly unstable, losing their total activity within 24 h at -20°C. Under similar conditions the partially purified enzyme from Sephadex G-150 column was stable, indicating that some stabilizing factor(s), most likely phospholipids, necessary for the maintenance of the biologically active conformation of the enzyme was lost during enzyme purification by ion-exchange column. This indeed was the case, as is evident from the results obtained on addition of phosphatidylcholine. Addition of phosphatidycholine restored nearly 46% of the activity of the enzyme,

which had earlier lost about 90% of its activity during storage at -20°C. These results also suggest that the PM- and GA-AMPases occur as complex of phospholipid and protein. The stability of the purified enzymes was greatly enhanced by 20% glycerol. Thus, the PM-AMPase and GA-AMPase show a great degree of resemblance in their properties. However, the two AMPase, besides having different molecular masses, vary in their immunological response. The polyclonal monospecific antibodies raised against the purified PM-AMPase did not cross react with the purified GA-AMPase as shown by Western blotting, double immunodiffusion and immunoprecipitation experiments. In other words the PM-AMPase and GA-AMPase do not have common determinants and may be structurally unrelated, being most likely the products of two different genes. The GA-AMPase may be a Golgi resident protein which does not migrate to the PM while the PM-AMPase migrates to PM by a default mechanism without being retained in GA.

The peanut PM-AMPases antibodies also did not cross react with detergent-solubilized AMPases from PM of other plant sources, for example, soybean, chickpea, wheat and pea. Thus, the plant AMPase are both subcellular as well as species-specific in nature. It is proposed that the GA-AMPase and PM-AMPase may be used as model glycoproteins for studying the intracellular transport of proteins in plant cells.

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Abbreviations

5′-ADP	Adenosine 5′- diphosphate
5′-AMP	Adenosine 5′- monophosphate
AMPase	Adenosine monophosphatase
5′- ATP	Adenosine 5′- triphosphate
B.P.B.	Bromophenol blue
^0C	Degree centigrade
cf	Cited from
CHAPS	(3-[(3- Cholamidopropyl)-dimethyl ammonio] - 1 - propane sulfonate)
Ci/mol	Curie/mole
cm	Centimeters
cpm	Counts per minute
DEAE-cellulose	Diethyl amino ethyl - cellulose
EDTA	Ethylene diamine tetra acetic acid
endo-H	Endo - N - acetyl - β - D - glucosaminidase - H
ER	Endoplasmic reticulum
Fig	Figure
g	Gram
GA	Golgi apparatus
GA-AMPase	Golgi apparatus-adenosine monophosphatase
5′- GDP	Guanosine 5′- diphosphate
5′- GMP	Guanosine 5′-monophosphate
GMPase	Guanosine monophosphatase
h	Hours
IDPase	Inosine diphosphatase
K_m	Michaelis - Menten constant
Kd	Kilo daltons
log	Logarithm

M	Molar
mA	Milli ampere
mg	Milli gram
min	Minute
ml	Milli litre
mM	Milli molar
nm	Nanometer
M_r	Molecular mass
MTase	Mannosyl transferase
µg	Microgram
µl	Micro litre
µC	Micro curie
µ mole	Micro mole
p -NPP	p-Nitrophenyl phosphate
Pi	Inorganic phosphate
PM	Plasma membrane
PM - AMPase	Plasma membrane -adenosine monophosphatase
ppm	Parts per million
RER	Rough endoplasmic reticulum
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second
SER	Smooth endoplasmic reticulum
TEMED	N,N,N',N',- Tetramethyl ethylenediamine
5'-UMP	Uridine 5'- monophosphate
5'-UDP	Uridine 5'- diphosphate
UMPase	Uridine monophosphatase
V_{max}	Maximum velocity
v/v	Volume / volume
w/w	Weight / weight
w/v	Weight / volume

Chapter 1

1.0 INTRODUCTION

5'-Nucleotidase (EC 3.1.3.5) catalyzes the hydrolysis of phosphate esterified 5'-carbon of the pentose moiety of the mononucleotide molecules. The enzyme is widely distributed in vertebrate tissues and has been reported to occur in bacteria and plants as well (Zimmermann, 1992). A large body of data is available in literature on this enzyme from animal tissues which indicates significant differences in their kinetic properties and substrate specificities. In addition, the enzyme has been shown to occur in different cellular locations i.e. in cytosolic, membrane-bound (transmembrane) (Zachowski, et al., 1981) and surface located (ectoenzyme) (Evans and Gurd, 1974) .This raises an interesting question as to whether the 5'-nucleotidases at different polygenetic levels or cellular locations belong to one major type of enzyme or they all are different enzymes. Although there is a great degree of variation in the homology of the enzymes from various sources (Zimmermann, 1992), the conserved character of protein structure is evident from comparable hydropathy profiles of different enzymes (Burns and Beacham, 1986; Volknandt et al., 1991; Zimmermann, 1992). Further both immunological and biochemical evidence show that cDNA isolated so far, corresponds to the membrane-bound and surface located forms of the enzyme. It remains to be seen if there exist tissue specific gene products. It may be noted that most of the data mentioned above are derived from vertebrates and little work has been done on the structural and functional relationship of plant nucleotidases.

Like animal 5'-nucleotidases, plant plasma membrane enzyme is a glycoprotein (Sharma et al., 1986) anchored to the membrane indicating a potential ectolocation, but the surface location has not been clearly established. The intracellular forms have been implicated in the catabolic pathways leading to ureides (Christensen and Joachimsen, 1983), cytokine metabolism (Chen and Kristopeit, 1981; Burch and Stuchbury, 1986) and in the regulation of nucleotide pool size (Carter and Tipton, 1986). However, purification and characterization of various forms of enzymes in plants is necessary for understanding the precise functional and structural relationships.

In earlier studies from this laboratory, 5'-nucleotidase showing high substrate specificity towards 5'-AMP, was purified from plasma membrane and the Golgi apparatus of peanut cotyledons (Sharma et al., 1986; Mittal et al., 1988). The plasma membrane and the Golgi apparatus enzymes showed remarkable resemblance in kinetic properties and substrate specificity but differed in carbohydrate content. In both fractions multiple forms of the enzyme were present. The plasma membrane contained three isoenzymes while the Golgi apparatus contained only two forms as judged by DEAE-cellulose elution profiles. The third enzyme, present in low concentration in plasma membrane, was completely absent in the Golgi apparatus. This third enzyme was considered of interest as this may be translocating from the endoplasmic reticulum to the plasma membrane by default mechanism without going through the Golgi apparatus.

Chapter 2

2.0 LITERATURE - REVIEW

2.1 OCCURRENCE AND SUBCELLULAR LOCALISATION OF 5'-NUCLEOTIDASE

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) is an integral glycoprotein of plasma membrane. This ubiquitous enzyme is involved in purine degradation and catalyzes the dephosphorylation of a variety of 5'-nucleotides such as adenosine monophosphate and inosine monophosphate to corresponding nucleosides on the cell surface.

Several authors (Drummond and Yamamoto, 1971; Arch and Newsholme, 1978; Dieckhoff et al., 1986, a, b; Kreutzberg et al., 1986; Luzio et al., 1986,1987; Widnell et al., 1986; Grondal and Zimmermann, 1988; Zimmermann, 1992) have reviewed various aspects of 5'-nucleotidase, such as, molecular properties and functions.

5'-Nucleotidase has been reported in various plant and animal sources. Extensive work has been done on animal system, such as, bovine semen (Bodansky and Schwartz,1963), rat liver (Fritzson,1969), chicken liver (Itoh et al., 1978), rat heart (Naito and Lowenstein, 1981), pigeon heart (Skladanowski and Newby, 1990), rat glioblastoma cell line (Turnay et al., 1992), dog heart (Darvish and Metting, 1993), calf thymus (Pesi et al., 1994). In plants this enzyme has been studied in tobacco pollen (Matousek and Tupy, 1984), peanut cotyledons (Sharma et al., 1986; Mittal et al., 1988), corn microsomes (Carter and Tipton, 1986) and soybean root nodules (Ostergaard et al., 1991). Willadson et al. (1993) studied the enzyme in *Boophilus microplus* and Itoh (1994) studied the enzyme from yeast.

Studies show that the enzyme is not distributed homogeneously among cellular elements of a given tissue or on the surface of an individual cell. 5'-Nucleotidase was detected at surface of endothelial cells and in hepatoma cells of rat (Van-den-Bosch et al., 1986), smooth muscle plasma membrane of dog ileum (Kostka et al., 1987), surface of Schwan and Satellite cells, plasmalemma, pinocytotic vesicles of endothelial cells and synaptic clefts (Nacimiento et al., 1991), myoblast cell surface (Mehul et al., 1993) and at various other sites.

Various groups reported the enrichment of enzyme activity in plasma-membrane than in other organelles (Emmelot et al., 1964, in rat liver; Perdue and Sheider, 1970, in chicken embryo fibroblasts; Flynn et al., 1986, in diatom *Phaeodactylum tricornutum*; Turnay et al., 1992, in rat glioblastoma cell line). Song and Bodansky (1967) and Widnell (1972) demonstrated the association of 5'-nucleotidase with the endoplasmic reticulum in rat liver. The association of enzyme with the Golgi apparatus was also shown in rat liver (Arsenius and Touster, 1968; Farquhar et al., 1974) and peanut cotyledons (Mittal et al., 1988).

Various groups suggested the ecto-enzyme nature of 5'-nucleotidase (Williamsons and Dipierto, 1965; Trams and Lauter, 1974; Dornand et al., 1982). Matsuura et al. (1984) reported the presence of both surface located and intracellular 5'-nucleotidase in rat liver. It is reported that whereas cytosolic enzyme activity controls intracellular levels of nucleoside 5'-monophosphates, surface located 5'-nucleotidase is major contributor to the cascade that completely hydrolyzes extracellular ATP to adenosine. Almost 50% of the enzyme resides intracellularly in membrane-bound pool depending on cell type. Major contributors are endocytotic pool including lysosomes (Maguire and Luzio, 1985; Wada et al., 1987) or also transcytotic vesicles. The continuous recycling of enzyme from the surface to an intracellular pool and back has been shown by cell

surface modulation in rat cultured hepatocytes (Stanley et al., 1980) fibroblasts (Widnell et al., 1982) or rat hepatoma cells (Van den Bosch et al., 1988). It is not known whether coated pits or some other endocytotic compartments are involved in endocytosis of glycosyl phosphatidylinositol anchored 5'-nucleotidase or not.

Various studies have suggested the presence of a glycolipid linker glycosyl phosphatidylinositol for membrane anchoring of membrane-bound 5'-nucleotidase (Stochaj et al. , 1989; Tanaka et al., 1989; Bailyes et al., 1990). It was found that in a variety of tissues and cellular systems the enzyme can, at least partially, be released from membranes by treatment with phosphatidylinositol specific phospholipase-C (Stochaj et al., 1989; Misumi et al., 1990). Further, the detergent solubilized and isolated enzyme was found to contain equimolar amounts of myo-inositol (Bailyes et al., 1990) which supported the notion of anchoring of enzyme to the membrane by glycosyl phosphatidylinositol. No information is available concerning the type of membrane anchorage or membrane topography in plants. The role of membrane anchorage by glycosyl phosphatidylinositol is not known but it may help in the release of proteins from glycosyl phosphatidylinositol anchor by endogeneous phospholipase (Fox et al., 1987), tight membrane packing of proteins or also production of free membrane lipids (Saltiel, 1990). It may act as a signal for targeting the attached protein selectively to the apical membrane surface (Lisanti et al., 1990).

Stanley et al. (1980), Widnell et al. (1982) and Van-den-Bosch et al. (1988) reported that surface derived 5'-nucleotidase can be differentiated from intracellular enzyme in rat hepatoma cells. There is a possibility that lysosome-endogenous phosphatidylinositol specific phospholipase -C is responsible for the conversion of the enzyme from membrane -bound to soluble form, as soluble form in rat liver was found to be devoid of glycosyl phosphatidylinositol anchor

and membrane-bound form could be released by phosphatidylinositol specific phospholipase-C. (Wada et al., 1987; Stochaj et al., 1989).

2.2 BIOSYNTHESIS AND GLYCOSYLATION OF 5'-NUCLEOTIDASE

The pathway of biosynthesis of 5'-nucleotidase is similar to other membrane proteins carrying glycosyl phosphatidylinositol anchor as shown by studies carried out in hepatoma cells (Van-den-Bosch et al., 1986; Misumi et al., 1990). The biosynthesis involves the synthesis of enzyme at rough endoplasmic reticulum and co-translational cleavage of signal peptide and core glycosylation. Van-den-Bosch et al. (1986, 1988) presented data on transport route of 5'-nucleotidase via the Golgi complex to plasma membrane in rat hepatoma cell lines. Ogata et al. (1990) and Volknandt et al. (1991) showed that mature 5'-nucleotidase lack terminal peptide extension and the hydrophobic amino acids on C-terminus get cleaved on post-translational modification and are exchanged for glycosyl phosphatidylinositol as a membrane anchor.

5'-Nucleotidase from plasma membrane was described as a glycoprotein in pig lymphocytes (Hayman and Crumpton, 1972), mouse liver (Evans and Gurd, 1974) and rat liver (Slavik et al., 1977). Effect of plant lectins also confirmed that enzyme is a glycoprotein (Turnay et al., 1992). Lectins bind to 5'-nucleotidase and inhibit its activity to varying degrees depending on tissue and cell type. Immunochemical studies showed the heterogeneity in carbohydrate moieties of 5'-nucleotidase from one cell type to another within the same species (Harb et al. 1985). Glycosylation and processing of carbohydrate chains was studied in cultured cells such as hepatoma cells (Van-den-Bosch et al., 1986), primary cultured hepatocytes (Baron and Luzio, 1987) and cell free system from rat liver (Wada et al., 1986). By differential treatment with endoglycosidase-H and endoglycosidase-F, the nature of glycan chain was differentiated. Tissue and species specific variations were found. Glycan chains

were reported to have hybrid (Harb et al., 1983) high mannose type (Meflah et al., 1984a) or complex type (Meflah et al., 1984b) of glycosylation. No evidence for O-linked glycosylation has been reported. Harb et al. (1983), Meflah et al. (1984) and Wada et al. (1986, 1987) showed that 5'-nucleotidase is a sialo-glycoprotein.

2.3 PATHWAY OF 5'-NUCLEOTIDASE ACTION

5'-Nucleotidase is involved in catabolism of mononucleotides to produce nucleosides. In animals, surface located enzyme is mainly involved in extracellular hydrolysis of 5'-AMP to adenosine (Gordon et al., 1986; Richardson et al., 1987; Terrain et al., 1989). As ecto-enzyme is inhibited by ATP and ADP, production of extracellular adenosine from AMP will be profoundly delayed on ATP release. This shows the restraint of extracellular adenosine formation as long as higher concentration of ATP (and ADP) are active in interstitial space. The whole mechanism is regulated by the enzymatic machinery which consists of 5'-nucleotidase, adenosine kinase, adenosine deaminase etc., working in close association and a substrate cycle between AMP and adenosine is produced (Arch and Newsholme, 1978). Cytoplasmic 5'-nucleotidase is involved in ATP degradation via AMP to adenosine or via IMP to inosine. The AMP concentration should be under control to prevent free interconversion of AMP, ADP and ATP by adenylate kinase as well as excessive activaiton of glycogen metabolism and glycolysis. ,The IMP preferring cytosolic forms appear to be present in all tissues although in varying concentrations (Itoh et al., 1986; Itoh and Yamada, 1991). In chicken, cytosolic dephosphorylation of IMP is first step in the pathway of uric acid formation from IMP (Tsushima, 1986). There is a possibility that production of inosine rather than of adenosine would avoid undesired physiological effect of adenosine (Itoh and Yamada, 1991).

Tozzi et al. (1991) proposed that enzyme mechanism involves the formation of a phosphorylated enzyme as an intermediate which can transfer the phosphate group either to water or to the nucleoside. Smolenski et al. (1994) reported that in normal conditions in human umbilical vein endothelial cells the adenosine is generated continuously, but it is immediately recycled via adenosine kinase.

2.4 PHYSIOLOGICAL ROLE OF 5'-NUCLEOTIDASE

The physiological function of the enzyme differs in various organisms and tissues and possibly extends beyond its catalytic activity. Klaushofer and Bock (1972) found direct relationship between cell proliferation and level of 5'-nucleotidase activity. Skladanowski and Newby (1990) predicted that enzyme gets activated during ATP catabolism and hence generates more than linear increase in adenosine formation rate in response to an increase in cytosolic AMP concentration. Stochaj and Mannherz (1990) pointed to a possible function of ecto 5'-nucleotidase as a cell receptor for extra-cellular matrix proteins. Sun et al. (1975) reported that enzyme activity in senescent human embryonic lung increases when it grows from young to mature stage. The intracellular forms of enzyme are associated with catabolic pathways, viz. in cytokine metabolism (Chen and Kristopeit, 1981), pathways leading to ureides (Christensen and Jochimsen, 1983; Burch and Stuchbury, 1986) and in regulation of the nucleotide pool size (Carter and Tipton, 1986).

Surface located 5'-nucleotidase is involved in inactivation and catabolism of ATP and formation of adenosine (Gordon et al., 1986; Richardson et al., 1987 and Terrain et al., 1989). The extracellular inactivation of ATP represents a necessary step in its control as an intracellular mediator. Adenosine induces a variety of physiological functions including vasodilation, a decrease in glomerular filtration rate, inhibition of urine release and neurotransmitter release, inhibition of inflammatory response or lipolysis (Stone, 1981; Gordon, 1986; Savic et

al., 1990, 1991; Daval et al., 1991). Itoh and Yamada (1991) implied a relation of enzyme activity to DNA synthesis in mammals. The low activity of enzyme results in toxic concentration of various nucleotides giving rise to rapid proliferation of malignant cells. This may be due to the increased DNA material. Nacimiento et al. (1991) reported that since distribution of enzyme in glial element and synapsis is quite typical for developing central nervous system, it may reflect specific cellular requirement for nucleosides involved in parenchymal metabolism, in vascular transport process and possibly in synaptic plasticity. Zhang et al. (1991) suggested that enzyme present on the luminal surface of superficial cells may play a special role in the membrane movement of these cells in the transitional epithelium.

Alterations of 5'-nucleotidase levels has been recognized in a considerable number of diseases (Sundermann, 1990). It has been reported that enzyme is either absent or present in very low level in malignant cells (Quagliata et al., 1974; Castle and Chibber, 1982). Various studies have been performed to discover any possible relationship between enzyme expression and cell phenotype and thus to evaluate the usefulness of cytochemical reaction in distinguishing different neoplastic lymphoid subpopulation in chronic malignancies. Invernizzi et al. (1990) confirmed the usefulness of 5'-nucleotidase cytochemical reaction for identification of lymphoid populations at different stages of maturation in chronic cell disorder. Libermann and Gordan-Smith (1980) correlated the myoproliferative and lymphoproliferative disorders in acute lukemia and Hodgkin's disease with 5'-nucleotidase activity.

2.5 REGULATION AND INHIBITION OF 5'-NUCLEOTIDASE ACTIVITY

5'-Nucleotidase is involved in a number of functions which presumably do not mutually exclude each other. They rather supplement each other in their contribution to the physiology of cellular metabolism and tissue function.

Several factors like chelating agents, nucleotides, divalent cations and phospholipids affect enzymatic machinery to produce a cumulative effect. In plants and animals both, the enzyme activity partially depends on the oxygen-carbon dioxide gas phase (Vincent et al., 1982). Several authors (Dornand et al., 1978; Chatterjee et al., 1979; Cammer et al., 1980; Naito and Lowenstein, 1981; Harb et al., 1984; Fini et al., 1985; Turnay et al., 1992) suggested that 5'-nucleotidase is a metalloenzyme since it is inhibited by EDTA and inhibition may be reversed by Mg^{2+} and Zn^{2+}. Other divalent cations, among them Ni^{2+} and Cu^{2+}, inhibit several 5'-nucleotidases (Murray and Friedrichs, 1969; Dornand et al., 1978). ATP has been reported to induce cooperative effects in sheep brain enzyme (Ipata, 1968) and activate rat liver enzyme (Van-den-Berghe et al., 1977). It has been postulated that the NTP inhibition of sheep brain enzyme may regulate the purine metabolism through AMP, thus the decrease of ATP in hypoxia situations (Baer et al., 1966) or in case of massive fructose administration (Schwarzmeier et al., 1977) would be the cause of the increase in purine catabolism. Mallol and Bozal (1983) reported that bovine brain 5'-nucleotidase is progressively and irreversibly inhibited by EDTA. The purines (except xanthine, 0.3 mM), pyrimidines and their nucleosides do not affect enzyme activity. The nucleoside di and tri-phosphates are competitive enzyme inhibitors against 5'-AMP as substrate. Nucleotide inhibition could be reversed by Mg^{2+}. Regulation of 5'-nucleotidase by cyclic nucleotides was suggested by Polya (1974) and Savic et al. (1991). Skladanowski and Newby (1990) showed that pigeon heart enzyme was inhibited by p-nitrophenyl phosphate and Pi, high Mg^{2+} concentration and high ionic strength. Decavandate has been found to increase the affinity of the soluble 5'-nucleotidase for 5'-IMP (Le Hir, 1991). Itoh (1994) reported that in yeast no detectable enzyme activity was noted with purine and pyrimidine nucleotides including AMP and dIMP but some activity was found for IMP. Mg^{2+}, Co^{2+} and Mn^{2+} were found to activate the enzyme. Lectins (concanavalin-A), have also been reported to inhibit

the enzyme activity (Carraway et al., 1976; Meflah et al., 1984,a; Flocke and Mannherz, 1991). Inhibition of enzyme by fibronectin and stimulation by laminin has been reported (Olmo et al., 1992; Mehul et al., 1993).

Various endogeneous inhibitors of 5'-nucleotidase have been reported in plants and bacteria. Polyphenolic substances which possess antitumor activity and inhibit 5'-nucleotidase have been isolated from seeds of *Areca catechu* (betel-nuts) (Iwamoto et al., 1991). As murine macrophage are directly stimulated by these inhibitors and no general cytotoxic effects were observed, the antitumor activity might be through potentiation of immunity of host animals (Matsuo et al., 1989). The presence of specific proteinaceous inhibitors was reported in cytoplasm of a number of bacteria (Dvorak et al., 1966; Neu, 1967) and in lymphocytes (Sun et al., 1983).

2.6 BIOCHEMICAL PROPERTIES

The biochemical properties of 5'-nucleotidase vary from source to source. The variations in the experimental procedures may account for some of the differences. Plant enzymes generally display marked substrate specificity. The relative rate of hydrolysis of major 5'-nucleotidases differ in a random manner. *E. coli and S. sonnei* possess nucleoside diphosphosugar hydrolase activity and also hydrolyse ATP and convert UDPG to base, sugar and inorganic phosphate (Neu, 1967,a,b; Neu, 1968). *Bacillus subtilis* does not hydrolyze ATP and converts UDPG to sugar phosphate (Dvorak et al., 1966). Best substrate for enzyme from hepatocyte and caudate nucleus membrane is purine nucleotides, particularly 5'-AMP (Meflah et al., 1984b) while liver lysosomal enzyme hydrolyzes both 2' and 3'-phosphates in addition to 5'-phosphates. The membrane-bound 5'-nucleotidase from *Halophile vibrio costicola* was responsible for hydrolysis of ATP, ADP and AMP (Benguis-Garber and Kushner, 1982). The *Boophilus microplus* enzyme can hydrolyze a range of nucleoside 5'-mono, di and

triphosphates to nucleosides. Limited peptide sequencing shows similarities to both mammalian 5'-nucleotidase and *E. coli* uridine diphosphate sugar hydrolase. It hydrolyzes UDPG, through a different mechanism than bacterial enzyme. It resembles mammalian nucleotidase in that there is evidence that it is attached to cell membrane by a GPI anchor. (Willadson et al., 1993). Potato 5'-nucleotidase has high activity towards p-nitrophenyl pyrophosphate (Polya, 1975). Membrane-bound 5'-nucleotidase from peanut cotyledons is higly specific for AMP (Sharma et al., 1986; Mittal et al., 1988). Higher plant enzyme is competitively inhibited by cyclic nucleotides (Polya, 1975; Carter and Tipton, 1986) and non-competitively by nucleosides. Generally, there is preference for nucleoside 5'-monophosphates and AMP is a preferred substrate. There may also exist the significant hydrolysis of 3'-AMP (Polya, 1974, 1975; Carter and Tipton, 1986) or of ADP and ATP (Chen and Kristopeit, 1981). 5'-nucelotidase from *Zea mays* seedlings has stronger affinity for purine nucleoside monophosphates than for pyrimidine nucleotides (Carter and Tipton, 1986). Generally, as reported, for membrane-bound 5'-nucleotidases, 5'-AMP is a preferred substrate. For cytosol enzyme the preferred substrate is IMP (Van-den-Bergh, 1977).

Plant 5'-nucleotidase have been found to show no absolute metal ion requirement (Polya, 1975; Sharma et al., 1986; Carter and Tipton, 1986; Mittal et al., 1988). Yeast enzyme is activated by Co^{2+} and Ni^{2+} (Takei et al., 1969). Animal 5'-nucleotidase has been reported to be activated by one or more of cations. Bull seminal plasma, chicken gizzard, snake venom, hepatocyte and caudate nucleus membrane 5'-nucleotidase have been reported to be metalloproteins (Meflah et al., 1984 b; Fini and Cannistrado, 1990).

For plants, native molecular mass varies between 50 to 69Kd but wheat germ contains additional form of 110Kd and maximum activity is at acidic pH (5-6). K_m values for AMP vary from millimolar to low micromolar values.

Animal nucleotidase vary markedly as molecular weight vary from 10Kd for snake to 265Kd for vertebrate heart. The pH range is from 5.0 to 8.0.

Pieri et al. (1991) reported that membrane microviscosity modulates the enzyme activity in liver plasma membrane of aging rats. With the increase in microviscosity, 5'-nucleotidase activity decreases. Baracca et al. (1994) reported that membrane fluidity does not influence the 5'-nucleotidase activity. It is suggested that the activity is sensitive to the amount of a specific fatty acid of the membrane (i.e. oleic or arachidonic acid) and/or to lipid supplementation which can influence the eicosanoids metabolism. It has been reported that like other GPI-anchored proteins, 5'-nucleotidase can be solubilized effectively solely with detergents of high critical micelle concentrations (octylglucoside, CHAPS or sodium deoxycholate) (Hooper and Turner, 1988). There is a possibility that 5'-nucleotidase has a membrane-integral anchor. However, after detergent solubilization, purification and reconstitution into phospholipid vesicles, membrane-bound 5'-nucleotidase (from chicken gizzard or human pancreatic carcinoma cells) is completely transformed into a hydrophilic form by GPI-specific phospholipase C or D (Stochaj et al., 1989 b). It is possible that the phospholipid anchor rather than a portion of the polypeptide chain is responsible for the 5'-nucleotidase and detergent interaction (Zimmermann, 1992). It is also possible that on enzyme isolation, the modification in the structure of anchor or the steric hinderance weakens the attack of GPI-specific phospholipase and thus release of GPI-bound 5'-nucleotidase in intact membranes.

2.7 PURIFICATION OF 5'-NUCLEOTIDASE

5'-Nucleotidase has been purified from a wide range of tissues in animals. Various procedures have been discussed for purifying 5'-nucleotidase from various sources (Dieckhoff et al., 1985; Fenvielle et al., 1987).

Various groups purified 5'-nucleotidase from different sources using different methods. Neu and Heppel (1964) found that 5'-nucleotidase of *E. coli* was released into solution when spharoplasts were prepared with ethylenediamine tetra acetate (EDTA)-lysozyme. Enzyme is also released from *E. coli* by osmotic shock. In this, cells in exponential growth phase are suspended in a medium of low ionic strength (Heppel, 1967). Osmotic shock has been used to release the enzyme from various enterobacteriaceae: *Shigella sonnei, Salmonalls heidelberg,* and *Proteus vulgaris* (Neu, 1967a; Neu, 1967b). Enzyme has been partially purified (70-fold) from 38,000 xg supernatant fluid from sheep brain homogenates by Ipata (1967, 1968). Widnell and Unkeless (1968) obtained a highly purified enzyme preparation from rat liver microsomes and plasma membranes using classical fractionation procedures in the presence of detergent. Various detergents have been used to solubilize the membrane-bound enzyme. Major criterion for selection of detergent is the retention of enzyme activity after solubilization and binding assay requires additional consideration. In general, digitonin, CHAPS and octylglucoside have been quite a popular choice. Bailyes et al. (1981) reported that Zwittergent 3 -14 was the detergent most capable of solubilizing 5'-nucleotidase. Meflah et al. (1984a) showed that 5'-nucleotidase was best solubilized by Zwitterrionic detergent sulfobetaine -14. Turnay et al. (1992) used Triton X-100 and CHAPS for solubilizing enzyme from rat glioblastoma cell line. Hjerten et al. (1988) used G3707 for extraction of a 5'-nucleotidase from *A. laidlawii* membrane and for removal of SDS. The enzyme activity has been reported to increase after removal of SDS.

Extensive work has been done to purify the enzyme using combination of density gradient centrifugation, differential centrifugation and column chromatography. Ivanov and Profirov (1981) isolated plasma membrane of high degree purity about (70%) from Ram spermatozoa using hypotonic EDTA medium and two phase polymer system of dextran-polyethylene glycol. Dieckhoff et al. (1985)

extracted 5'-nucleotidase from chicken gizzard smooth muscle using sulfobetaine derivative of cholic acid and purified to homogeneity by employing three chromatographic steps. Sharma et al. (1986) and Mittal et al. (1988) purified enzyme from the plasma membrane and the Golgi apparatus fractions by selective solubilization using octylglucoside and ion-exchange chromatography on DEAE-cellulose column. Flocke and Mannherz (1991) also used two affinity chromatographic steps for purification of enzyme. Darvish and Metting (1993) purified enzyme from 150,000 xg supernatant using phosphocellulose, DEAE-cellulose and ADP-agarose affinity chromatography. Ann and Snow (1993) described 8% (v/v) butanol extraction procedure to provide 3-fold purification and enzyme preparation for further purification. Flynn et al. (1987) described two polymer phase system to partition 5'-nucleotidase into upper phase and chlorophyll into lower phase in diatom *Phaedactylum tricornutum* microsomal preparation. Ellis et al. (1992) used sucrose density gradient centrifugation and differential centrifugation to get 21-fold purification of enzyme in apical membrane preparation.

2.8 CURRENT STATE

In summary, the literature shows that to obtain subcellular membrane fractions from plant tissues in active and pure forms is comparatively difficult due to presence of rigid cell wall and lack of convenient markers. In general dissolution of cell wall by hydrolytic enzymes or treatment with cellulase has been used to soften or remove the cell wall in yeast and bacteria (Lehle, 1980) while in higher plants the tissue is homogenized using razor blade chopping, pestle and mortar or glass teflon homogenizer in a buffered medium, usually Tris (pH 7.0 to 8.0) with sucrose (0.25 to 0.5 M) as osmoticum, EDTA (1-3 mM, to chelate divalent cations and convert rough ER to smooth ER) and sometimes MCE, DTT or cysteine (to inhibit phenolic oxidation and to improve the long-term stability of enzymes during the isolation procedure). Mg^{2+} is used to obtain

the intact ribosomes as it reduces the ribonuclease activity and glutaraldehyde helps in stabilization and production of active Golgi apparatus.

After homogenization, homogenate is filtered through mesh and differential centrifugation is done to remove cell debris and larger organelles. Low speed centrifugation (150-2500 xg) sediments heavy particles like nuclei, 10,000 xg centrifugation pellets mitochondria and chloroplasts and centrifugation at 100,000 xg pellets microsomal membrane fraction containing plasma membrane, Golgi apparatus and endoplasmic reticulum. Density gradient centrifugation is performed using materials like sucrose, serum albumin, Ficoll (Leonard and Vanderwounds, 1976) and silica gel to isolate and purify different subcellular fractions. However, the method of purification is modified as per requirement.

A 20-50% sucrose density gradient has been used (Hall, 1983; Bonner, 1965; Marriott, 1975) for purification of plasma membranes from the fractions obtained by differential centrifugation. The peak equilibrium density for plasma membrane in sucrose generally lies between 1.14 and 1.17g/cm^3 (Leonard and Hodges, 1980) but lower values have also been reported. With the help of precisely prepared density gradients together with marker enzymes, it is possible to prepare highly enriched membrane fractions from plant sources. Recently Muller et al. (1994) reported that it was possible to selectively enrich GPI-proteins upto 8 to 14 fold by using concentrations between 0.1 and 0.3% of 4-NH_2-amino-7-beta benzamido-taurocholic acid (BATC).

Although, the plasma membrane has been the subject of physiological and biochemical research for decades, only recently has the molecular analysis of plasma membrane proteins have been achieved (Sussman and Harper, 1989). cDNA and genomic clones encoding the primary active pump, the proton pump, have been isolated and sequenced. DNA clones for other plasma membrane

proteins like 5'-nucleotidase will be useful in evaluating cellular, developmental or hormone-specific expression of isoforms. The purification and characterization of a glycopeptide with AMPase activity reported in this thesis may lead to molecular biology studies of the plant plasma membrane.

Chapter 3

3.0 MATERIALS AND METHODS

3.1 MATERIALS

GDP-^{14}[C] mannose was purchased from Radiochemical Centre, Amersham (U.K.). Tris, CHAPS (3-[3-cholamidopropyl - dimethyl ammonio]-1-propane - sulfonate), bovine serum albumin, adenosine monophosphate, DEAE - cellulose, Sephadex G-150, SDS, (sodium lauryl sulphate), Freund's adjuvants, molecular weight standards were purchased from Sigma Chemical Company (St. Louis, USA). Acrylamide, N, N'-methylene - bis -acrylamide and TEMED were obtained from Serva (FRG). β-mercaptoethanol and ammonium persulphate were purchased from E-Merck. The protein-A insoluble from *Staphylococcus aureus* was acquired from Fluka, Switzerland. All other chemicals were reagent grade from standard commercial firms. Peanut (*Arachis hypogaea* L.) seeds (large variety) were obtained locally.

3.2 METHODS

3.2.1 Germination Of Seeds

Peanut seeds were surface sterilized for 15-20 min, followed by thorough washing with distilled water to remove the disinfectant. These seeds were then germinated on a moist, 4 layered pre-sterilized cheese-cloth in dark for specified number of days at 35°C in a seed germinator under aseptic conditions. The seeds were washed twice every day with distilled water to prevent fungal infection. Whenever germination was not necessary, the seeds were imbibed by immersing them in sufficient amount of water for 12h and then used for further experimentation.

3.2.2 Buffers Used

Buffer - A 50 mM Tris - HCl, pH 7.2, containing 0.25 M sucrose, 3 mM EDTA and 0.01% (v/v) β-mercaptoethanol.

Buffer - B 25 mM Tris - HCl, pH 7.2, containing 20% (w/v) sucrose and 1 mM β-mercaptoethanol.

Buffer - C 25 mM Tris - HCl, pH 7.2, containing 1 mM β-mercaptoethanol.

Buffer - D 50 mM Tris - HCl, pH 7.2, containing 0.25 M sucrose, 0.04% (v/v) β-mercaptoethanol, 20 mM $MgCl_2$ and 5 mM EDTA.

Buffer - E 10 mM Tris - HCl, pH 7.2, containing 1 mM β-mercaptoethanol and 0.01% CHAPS.

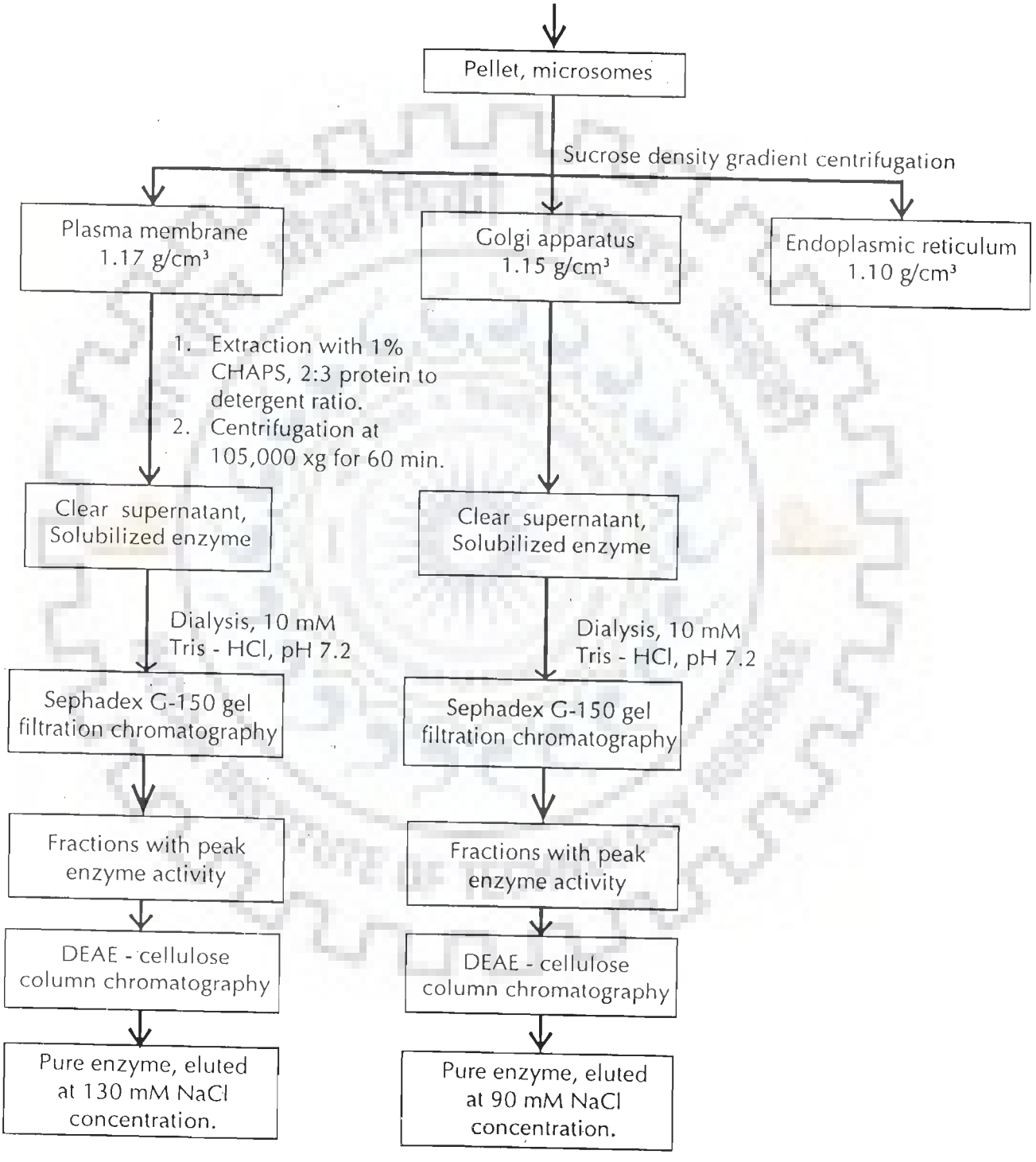
Buffer - F 10 mM Tris - HCl, pH 7.2, containing 1 mM β-mercaptoethanol, 0.01% CHAPS and 20% glycerol.

3.2.3 Plasma Membrane Isolation

Unless stated otherwise all operations were carried out at 0-4°C. Membrane fractions were prepared from germinating cotyledons as described by Sharma et al. (1986). Briefly, peanut cotyledons (200 in number and 80 - 100 g fresh weight) were excised, rinsed in deionized water and blended in 200 ml buffer-A for 30 sec. The homogenate was filtered through 8 layered cheese-cloth and centrifuged at 12,000 xg for 20 min, followed by centrifugation at 135,000 xg for 60 min. The 12,000 - 135,000 xg microsomal pellet was suspended in 4 ml buffer-B, layered carefully over 5 ml of 34% (w/v) sucrose in buffer-C and centrifuged at 80,000 xg for 90 min (Fig. 3.1). The clear sucrose layers were removed carefully by aspiration. The endoplasmic reticulum (ER) plus Golgi apparatus (GA) fraction remaining at the interface of 34% and 20% sucrose and the pellet containing plasma membrane (PM) were recovered. The PM was collected and suspended in 2 ml of buffer-B.

Fig. 3.1 FLOW CHART FOR PURIFICATION OF AMPase FROM PM AND GA FRACTIONS

1. Homogenization of 7-days old germinating peanut cotyledons in Tris - HCl, pH 7.2, with 0.25 M sucrose, 3 mM EDTA and 0.04% β-mercaptoethanol.
2. Filtration through 4 layers of cheese - cloth and centrifugation sequentially at 12,000 xg for 20 min and at 105,000 xg for 60 min



The PM vesicles were separated from other membranes by a discontinuous sucrose density gradient centrifugation method as described by Hall (1983). The discontinuous gradients were prepared in 38 ml tubes by carefully layering 4 ml of 45% (w/w) sucrose and 6.4 ml each of 38%, 34%, 30%, 25% and 20% sucrose. 2 ml (25 mg) protein was carefully layered onto the gradient and centrifuged at 95,000 xg for 2h. The PM fraction at 47/38% interface corresponding to an equilibrium density in sucrose of 1.15-1.17 g/cm^3 was identified by the presence of marker enzyme 1,3-β-D-glucan synthase (Hall, 1983) and used further.

3.2.4 Separation Of Golgi Membranes From The Endoplasmic Reticulum

The membrane fraction banding at the interface of 34/20% (w/w) sucrose, obtained from the above step, was used for the preparation of Golgi apparatus (GA) and endoplasmic reticulum (ER) by sucrose density gradient method of Green (1983) with minor modifications. For the separation of the GA from the ER, the interface membrane fraction was diluted to about 10% sucrose by buffer-C and pelleted by centrifugation at 135,000 xg for 60 min. The pellet was resuspended in 4 ml of buffer-B and layered carefully onto a 3-step sucrose density gradient system composed of 7 ml each of 43%, 37% and 25% sucrose successively in the same buffer in 25 ml tubes and centrifuged at 135,000 xg for 180 min in Centrikon-T-2060 centrifuge using the TFT 70.38 Centrikon rotor. The membrane fraction banding at the interface of 37/25%, corresponding to a density of 1.15 g/cm^3, was recovered and diluted to 10% sucrose by buffer-C and pelleted by centrifugation at 135,000 xg for 60 min. The resulting pellet was then suspended in 2-3 ml buffer-B. The insignificant activity of 1,3-β-D glucan synthase and mannosyltransferase, the marker enzymes for PM and SER and high activity of IDPase, marker enzyme for GA indicated that this enriched GA fraction was free from cross contamination of PM and SER (Sharma et al., 1986).

The membrane suspension staying above the 37/25% interface corresponding to a density of 1.1 g/cm^3 was diluted to 10% sucrose with buffer-C and centrifuged at 135,000 xg for 60 min as before. The resulting pellet was then suspended in 2-3 ml of buffer-B.This fraction represented the enriched ER which was relatively free from the cross- contamination of PM or GA (Sharma et al., 1986) as shown by the distribution of the marker enzymes 1,3-β-D-glucan synthase (Hall,1983), inosine diphosphatase (IDPase)(Green, 1983) and mannosyltransferase (Lord, 1983) for PM, GA and ER, respectively.

3.2.5 Solubilization Of Membrane-Bound AMPase

All steps of solubilization and purification were carried out at 0-4^{0}C. The membrane fraction (30 mg/ ml) was suspended in buffer-D with a teflon tissue homogenizer. To 1 ml suspension (30 mg protein) 1.0% CHAPS in same buffer was added dropwise with constant stirring so that the final detergent concentration and the protein to detergent ratio were 1% 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. After 30 min incubation in ice the homogenate was centrifuged at 135,000 xg in a Centrikon T-2060 centrifuge using the TFT 70.38 Centrikon rotor. The clear supernatant was collected and the enzyme activity was assayed. The pellet was resuspended in buffer-C containing 1% detergent and assayed for enzyme activity and protein content. In some cases the solubilized enzyme was stabilized by the addition of 20% glycerol (final concentration) in the solubilizing buffer.

3.2.6 Sephadex G-150 Column Chromatography

Sephadex G-150 (20 g) resin was suspended in 400 ml deionized water and allowed to swell for 5 -days in cold (0-4^{0}C). The supernatant and fine particles of the resin were removed. The washed resin was deaerated and packed in a glass column (1x100 cm). The column was equilibrated overnight with buffer-E in cold (0-4^{0}C).

The solubilized enzyme was dialyzed for 6 h against 100 volumes of buffer-E. The dialyzed enzyme was applied on a precooled Sephadex G-150 column. The absorbed proteins were eluted by using the same buffer. Fractions of 2 ml each were collected at flow rate of 1 ml/20min. Aliquots (0.1 ml) from every alternate fraction were analyzed for the protein content and enzyme activity. The peak enzyme containing fractions were pooled and further purified.

3.2.7 DEAE-Cellulose Column Chromatography

10 to 20 g of DEAE-cellulose was suspended in 500 ml of 0.25 M NaOH and allowed to swell for 30 min. It was then neutralized with HCl, filtered and finally rinsed with deionized water until neutral. pH was tested with pH paper. After that it was suspended in 500 ml of 0.25N HCl for 10-15 min, filtered and rinsed with deionized water. Then repeated NaOH wash, rinsed and suspended in buffer-E. The charged DEAE-cellulose was packed in a glass column (1.5x10 cm) and equilibrated overnight with buffer-E in cold (0-4°C) at flow rate of 0.2 ml/min.

The pooled enzyme from Sephadex G-150 gel filtration column was applied on the pre-equilibrated DEAE-cellulose column. The column was washed with 20 ml of buffer-F to remove unabsorbed proteins. The absorbed proteins were eluted by a linear gradient of 0 to 300 mM NaCl using a single mixing container with 50 ml buffer-F and reservoir with 50 ml of same buffer containing 300 mM NaCl. Fractions (2 ml) were collected at the flow rate of 0.2 ml/min. Aliquots (0.2 ml) from every other fraction were analyzed for protein content and enzyme activity. The peak enzyme containing fractions were pooled and analyzed further.

3.2.8 Polyacrylamide Gel Electrophoresis (PAGE)

3.2.8.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis was carried out in the presence of SDS by Laemmli's (1970) method using gel slabs (14x14x0.2 cm) with either 8 wells of about 1 cm

width and 4 mm spacing or 11 wells of about 0.6 cm width and 3 mm spacing in between two consecutive wells. All reagents were prepared in double distilled deionized water. Various solutions used were as follows:

Stock Solutions

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

Solution B - 1.5 M Tris-HCl buffer, pH 8.8, containing 0.4% (w/v) SDS.

Solution C - 0.5 M Tris-HCl buffer, pH 6.8, containing 0.4% (w/v) SDS.

Solution D - 10% (w/v) ammonium persulphate. This solution was prepared fresh before use.

TEMED - N,N,N',N' tetramethylethylene diamine.

10% SDS - 10 g of SDS in 100 ml double distilled water.

Working Solution For Preparation Of Gel

Separating gel

	8.0% (w/v)	10% (w/v)	12% (w/v)
Water	18.6 ml	15.8 ml	13.2 ml
Solution A	10.7 ml	13.3 ml	14.0 ml
Solution B	10.0 ml	10.0 ml	10.0 ml
10% SDS	0.4 ml	0.4 ml	0.4 ml
10% APS	0.4 ml	0.4 ml	0.4 ml
TEMED	0.024 ml	0.016 ml	0.016 ml

Stacking gel [5% (w/v)]

Water	6.8 ml
Solution A	1.7 ml
Solution B	1.25 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

Working solutions for separating and stacking gels were deaerated prior to the initiation of polymerization with ammonium persulphate.

Electrophoresis Buffer

0.025 M Tris, 0.192 M glycine and 0.1% SDS (pH 8.3).

Sample Preparation

Enzyme samples (100-200 μg protein) from different steps of purification were dialyzed against water, concentrated and dissolved in 100 μl of the sample buffer (62 mM Tris-HCl, pH 6.8, containing 1% SDS (w/v), 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol). This was followed by heating for 5 min in a boiling water bath. Molecular weight markers were also subjected to the same treatment. 20 μl of 0.6% bromophenol blue was added to all the tubes after cooling to room temperature. These proteins were then loaded in the wells and electrophoresis was carried out at constant current of 20 mA for stacking gel and 30 mA for the separating gel. When the tracking dye reached close to the base (1 cm from bottom) of the gel, the gel was removed and stained by immersing in 0.5% coomassie brilliant blue R-250 in methanol-acetic acid-water (25:10:65, v/v/v) for 2h at room temperature. Gel was destained by washing with destaining solution, methanol-acetic acid-water (25:7:68, v/v/v). Sometimes silver staining (Davis et al.,1986) was also done to locate the protein bands on the gel. Mobilities of different protein bands were determined relative to bromophenol dye using the following equation:

$$\text{Relative mobility} = \frac{\text{gel length before staining}}{\text{gel length after staining}} \times \frac{\text{distance travelled by protein band}}{\text{distance travelled by bromophenol blue}}$$

The R_f values of standard molecular weight markers were plotted against the log of molecular weights and the molecular mass of the purified enzyme was determined using this standard curve.

3.2.8.2 Native PAGE

The native gel electrophoresis was performed under non-reducing and non-denaturing conditions at a constant current of 30 mA at 4^0C. Unstained gel was sliced serially into 2 mm thick sections. Slices were homogenized in an aliquot (500 µl) of enzyme incubation buffer. Gel suspension was agitated at frequent intervals to facilitate maximum elution of protein and then centrifuged at 10,000 xg for 10 min. Enzyme assay was done by adding the substrate to the eluted fraction. The activity was expressed as µ mole of Pi liberated/min/gel slice.

3.2.8.3 Silver staining

Silver staining was done by the method of Davis et al. (1986). The gel was fixed in 50% methanol for 2 h on an orbital shaker. The silver staining solution was prepared just prior to use by mixing 21 ml of 0.36% NaOH with 1.4 ml of 14.8 M NH_4OH and then adding 4 ml silver nitrate solution (0.8 g $AgNO_3$) dropwise with vigorous vortexing. The solution was made upto 100 ml with deionized water. This staining solution was added to the gel followed by gentle shaking in the orbital shaker for 15 min. After this the gel was washed for 30 min in 3-4 changes of water on the orbital shaker and treated with freshly prepared developing solution (25 mg citric acid and 0.25 ml of 38% (w/v) formaldehyde in total volume of 500 ml). When the bands reached the desired intensity, the gel was washed briefly with water and reaction was stopped by agitating the gel in methanol-acetic acid-water (45:5:50, v/v/v). The stained gels were stored in this solution in dark.

3.2.9 Semi Dry Electrophoretic Transfer

The electrophoretic transfer was done by the method of Kyhse-Andersen

(1984). SDS-PAGE was run using the protein samples by method of Laemmli (1970) as described earlier. After the run was complete, the gel was taken out and kept in Tris- buffer (pH 9.0 to 9.4) (Tris, 5.82 g, 3.75 ml of 10% SDS, 2.93 g glycine and 200 ml of methanol - adjusted the volume to one litre). Twelve filter paper strips of the size of the gel were cut and one strip of nitrocellulose of same size was taken. These strips and nitrocellulose sheet were kept in buffer for 15 min. On the bottom of the plate of the apparatus (the anode), assembled the gel, nitrocellulose and filter paper strips in order: bottom electrode, six layers of filter paper, one nitrocellulose membrane, polyacrylamide gel, six layers of filter paper. Air bubbles were checked carefully and gently removed either by using gloved hand or by rolling a pipette over the sandwitch. Dried the buffer surrounding the gel paper sandwitch using tissue paper. Placed the upper electrode on the top of the stack. Connected the electrodes and commenced the transfer for 1h at 20V. After the transfer, disconnected the power source and marked membrane to follow the orientation. Stained the gel with coomassie brilliant blue R-250 or silver stain to verify the transfer. From the side of lane of molecular weight standards one strip of nitrocellulose membrane was cut, stained with 0.1% amidoblack (0.1 g amidoblack in 25% isopropanol and 10% acetic acid) for 5 min and then destained in 25% ethanol and 10% acetic acid. Strip was dried and stored. Rest of the nitrocellulose membrane was processed further.

3.2.10 Western Blotting

Amersham Western blotting kit was used.

Buffers used were :

(i). Tris buffer saline (TBS), pH 7.6 : 2.42 g Tris-base (20 mM), 8.0 g sodium chloride (137 mM), 3.8 ml 1 M HCl - diluted to 1000 ml with distilled water and checked the pH.

(ii). TBS Triton X-100 (TBS-T) : 1 ml of Triton X-100 (0.1%) in 999 ml TBS.

(iii). Diethanolamine buffer : 1.05 g diethanolamine (100 mM) and 100m g magnesium chloride hexahydrate (5 mM) were added to 90 ml double distilled water, dissolved and adjusted pH to 9.5 with 5 M hydrochloric acid and volume was made upto 100 ml.

Samples for electrophoresis were prepared. The positive control immunoglobulin was diluted 1:100 in electrophoresis sample buffer and boiled for 2 min. Electrophoresis was performed and afterwards the proteins were blotted on nitrocellulose sheet as described earlier.

After transfer, the membrane was blocked by immersing it in a 5% solution of dried milk membrane blocking agent in TBS-T. It was kept at room temperature for 1h on a shaker. During the membrane blocking stage, diluted the primary antibody in TBS. Briefly rinsed the membrane using two changes of TBS-T, then washed the membrane three times in fresh changes of TBS-T for at least 5 min each wash, at room temperature. Then the membrane was incubated with primary antibody at room temperature for 1h. Again the membrane was washed with TBS-T in the same manner. Biotynylated antibodies were diluted 1:500 in TBS and added to the membrane and incubated for 20 min at room temperature. Membrane was washed again with TBS-T and 1:3000 diluted streptovidin - alkaline phosphate conjugate was added to the membrane. Incubation was done for 20 min at room temperature. Washed the membrane with TBS-T and added enzyme substrate (nitro -blue tetrazolium in dimethyl formamide and 5-bromo-4-chloro-3- indolyl phosphate in dimethyl formamide-one drop each in 10 ml of diethanolamine buffer). Membrane was incubated with enzyme substrate at room temperature for 30 min. Washed the membrane with double distilled water. Dried the membrane between sheets of filter paper and photographed.

3.2.11 Immunological Studies

3.2.11.1 Production of antibodies

Polyclonal monospecific antibodies were raised against the purified enzyme in the NewZealand strain white female rabbits following standard protocol. Rabbits were bled before immunization from the marginal ear vein to obtain preimmune (control) serum and to check the titre of unwanted antibodies in the animal. The purified enzyme (400 µl, 1 mg protein) in phosphate buffered saline was thoroughly emulsified in equal volume of Freund's complete adjuvant until a stable emulsion was obtained as indicated by non-disruption of emulsion drop when added to water. This emulsion, containing 1.00 mg protein, was injected subcutaneously into eight sites on the animal's back (0.1 ml/site). The second injection containing 500 µg protein with equal volume of Freund's incomplete adjuvant was given 21 -days after the first injection. Same eight sites of injection were chosen. Second booster dose was given 7 -days after the first booster dose. 400 µg of protein in Freund's incomplete adjuvant was injected as described above.

3.2.11.2 Collection of antiserum

The rabbits were bled from the marginal ear vein 7 -days after the second booster injection. The blood was allowed to clot at room temperature for about 1 h and left overnight at 4°C. The clot was detached from the walls of centrifuge tube with the help of a thin wire giving circular motion to the wire while keeping the tube fixed. The clear, clot-free fluid was poured off into another centrifuge tube. The clot was centrifuged for 30 min at 3,000 xg at 4°C and any expressed fluid was removed by Pastuer pipette and combined with the previous one. The pooled liquid was centrifuged for 20 min at 2,000 xg at 4°C. The clear straw coloured serum was stored in aliquots at -20°C.

3.2.11.3 Test of antibodies

The antibodies formation against the purified enzyme was tested by carrying

out the immunoinhibition studies. The antiserum and preimmune serum were diluted to 1:5, 1:10, 1:20, 1:50 and ability to inhibit the enzyme activity was tested using standard enzyme assay procedure with varying concentration of antiserum. Controls contained an equal amount of preimmue serum. In classical experiments, 200 µl enzyme was incubated with 50 µl of the antiserum or preimmune serum of various dilutions for 4 h at 4°C. After incubation, 100 µl of the protein- A (Fluka) was added to each incubation and the mixture was further incubated for another 3 h at 4°C. The protein-A enzyme antibody complex was removed by centrifugation at 3,500 xg for 15 min at 4°C. The supernatant was assayed for the enzyme activity. Inhibition of the enzyme activity is taken as a measure of antibody formation against the enzyme.

3.2.11.4 Partial purification of antibodies

The antibodies (immunoglobulin G, IgG) were purified from the serum of immunized rabbits following the procedure of Mayer & Walker (1987). The serum was brought to 45% saturation of $(NH_4)_2SO_4$ by slow addition of 1.5 g solid $(NH_4)_2SO_4$ with gentle stirring. The protein precipitate was collected by centrifugation at 10,000 xg for 30 min, washed several times (4-5) with 1.75 M $(NH_4)_2SO_4$ until the colour of the precipitate was white. The washed precipitate was dissolved in 10 ml of 10 mM sodium-phosphate buffer (pH 7.0) and dialyzed overnight against water (500 ml) at 4°C. Any precipitate that appeared during dialysis was removed by centrifugation at 10,000 xg for 15 min. The clear supernatant containing IgG fraction was collected with the help of Pastuer pipette and dialyzed overnight against 10 mM sodium-phosphate buffer, pH 8.0, at 4°C. The dialyzed IgG fraction was loaded onto a DEAE-cellulose column (1x8 cm) previously equilibrated with 10 mM phosphate buffer, pH 8.0. Column was washed with equilibration buffer at a flow rate of 0.25 ml/min. Fractions (5 ml) were collected and monitored at 280nm. IgG was separated in the washings as unabsorbed protein, as an unsymmetrical peak (Fig.4.16). The protein containing fractions in the peak were

pooled and precipitated with $(NH_4)_2SO_4$ as before. The precipitate was collected and dissolved in 5 ml of 20 mM sodium-phosphate buffer, pH 7.0, containing 0.15 M NaCl. IgG from the preimmune serum was also purified in the same manner.

3.2.11.5 Double immunodiffusion

Immunodiffusion was performed by the method of Ouchterlony and Nilsson (1986) on 8x4 cm glass slides, coated with 1.5 mm layer of 1% agarose gel. 1.0 g agar was dissolved in 100 ml phosphate-buffered saline (PBS), containing 0.02% (w/v) sodium azide (PBS-azide), in the boiling water bath until completely dissolved. The agar solution was allowed to cool to 45-50°C. 5.5 ml of this solution was poured on each slide. The slides were kept on flat surface and then allowed to solidify at room temperature so that gels of 1.5 mm thickness were formed. In the centre of the agarose gel 10 µl well was punched out and four wells of 10 ml were punched out in a circular fashion at equal distances of 1.0 cm from it. The central well was filled with 10 µl of the purified enzyme (20 mg protein) and the surrounding two wells with antiserum and two wells with preimmune serum. The loaded gels were kept overnight (16-18 h) at 37°C in a humid plastic box. The appearance of white precipitin bands between the central well and surrounding wells containing immune serum indicated the presence of antibodies in the antiserum. Single precipitin band was indicative of the homogeneous antigen. Precipitin bands did not appear between the central well and the wells containing preimmune serum.

3.2.12 Membrane Labelling

The cotyledons (100 g) were homogenized with a mortar and pestle at 4°C in 0.1 M Tris-HCl (pH 7.4) containing 5 mM dithiothrettole, 10 mM $MgCl_2$ and 0.4 M sucrose. The homogenate was filtered through 4 layers of cheese-cloth. The filtrate was centrifuged at low speed (10,000 xg for 20 min). Supernatant was taken and the total membrane preparation was obtained by centrifugation at 135,000 xg

for 60 min. The membrane preparation was resuspended in homogenization buffer mentioned above at a protein concentration of approximately 2 mg/ ml and incubated with radioactive substrates (GDP-[^{14}C] man), final incubation concentration of 20 mM ($4x10^5$dpm), added to each incubation in 60 ml total volume, at 25°C.The reaction was terminated by using chloroform: methanol (1:1, v/v) to bring final concentration of chloroform:methanol:water to 10:10:3.

3.2.13 Product Characterization

Reaction mixture from membrane labelling was thoroughly washed with chloroform: methanol : water in 10:10:3 ratio (v/v, 8 ml) and the insoluble material was separated by centrifugation. The pellet obtained from centrifugation of initial extract with chloroform:methanol:water was washed with the same solvent (2x8 ml) followed by methanol (1x8 ml) and lyophilized. An aliquot was subjected to boiling after dissolving in 1 ml of 1.0 M Tris-HCl (pH 6.8) containing 2% SDS (w/v) and 10 mM DTT. SDS insoluble material was removed by centrifugation using an eppendorf microcentrifuge for 5 min and washed once with 1 ml SDS buffer at 100°C for 10 min. The pellet obtained after recentrifugation contains polysaccharide material while soluble product was glycoprotein. The radioactivity of SDS solubilized material was counted using LSC, then subjected to SDS-PAGE. Fluorography of gels was performed using X-ray films.

3.2.14 Estimation Of Total Carbohydrate Content

Total carbohydrate content was determined by phenol-sulfuric acid method given by Dubois et al. (1956). To a 2 ml aqueous solution (20-75 µg carbohydrate) 50 µl of 90% phenol solution and 5 ml of concentrated H_2SO_4 were added, followed by quick stirring on vortex. The mixture was incubated at room temperature for 30 min and the absorbance of the orange colour was measured at 485nm against the blank which was prepared without sample. Amount of carbohydrate was computed from the standard curve of glucose, prepared under identical conditions.

3.2.15 Enzyme Assays

3.2.15.1 AMPase assay

AMPase activity was measured with 5'-AMP as substrate as described by Riemer and Widnell (1975) with slight modifications. The standard reaction mixture, unless stated otherwise, contained (1 to 2.5 mM 5'-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 Pi was determined in the supernatant fluid by the procedure of Fiske and SubbaRow (1925). The specific activity of AMPase corresponds to the µ moles of Pi liberated by dephosphorylation of 5'-AMP per min per mg-protein under standard assay conditions. When detergents, especially Trition X-100 and Nonidet NP-40 were present in the assay system, the Pi was determined by the modified Fiske and SubbaRow method as described by Dulley (1975). In this method Pi analysis was done in the presence of 3% SDS which eliminated interference due to Triton X-100 and other detergents.

3.2.15.2 Assay for glucan synthase

The activity of 1,3-β-D-glucan synthase was measured as described by Ray (1977) using high concentrations of UDP-glucose in the absence of Mg^{2+} with slight modification. The standard incubation mixture, unless stated otherwise, contained the following in a final volume of 100 ml. Tris-HCl (50 mM, pH 7.4), UDP-[^{14}C] glucose (0.1µCi, 290 Ci/mol), 0.5 mM unlabelled UDP-glucose, 5 mM cellobiose, 2 mM $CaCl_2$, 0.01% digitonin and 0.02 to 0.1 mg protein, depending upon the status of purification of the enzyme. After incubation for 20 min at 25°C the reaction was terminated by the addition of 1 ml ethanol, 0.05 ml 50 mM $MgCl_2$ and 0.15 ml boiled plasma membrane (1.0-1.5 mg protein) as a carrier for the

labelled products. The mixture was immediately boiled for 1 min and after standing overnight at 4°C the polymer was separated by centrifugation at 3000 xg for 10 min. The pellet was washed four times with 70% (v/v) ethanol to remove unreacted radioactive substrate and ethanol soluble products. The washed precipitate was suspended in 5 ml scintillation fluid (dioxan cocktail) and radioactivity measured in a Beckman L.S. 1801 liquid scintillation counter. Control assays were performed exactly the same way except that an equivalent amount of boiled enzyme was used in place of active enzyme preparations.

3.2.15.3 Assay for IDPase

The IDPase activity was assayed as described by Green (1983). The assay mixture contained the following components in a final volume of 1 ml : 3 mM inosine diphosphate (IDP), 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) and 0.1 ml (100 mg membrane protein). After 60 min incubation at 20°C the enzyme reaction was terminated by the addition of 1 ml cold 10% trichloroacetic acid. Protein was removed by centrifugation and Pi was determined in the supernatant fluid by the method of Fiske and SubbaRow (1925).

3.2.15.4 Assay for mannosyltransferase

The mannosyltransferase activity was assayed by the method of Lord (1983). 1 ml of membrane fraction was adjusted to 10 mM $MgCl_2$ and the reaction was started by adding 0.02-0.2 μCi of GDP-[^{14}C] mannose, sp. act. 228 Ci/mol, at 30°C for 30 min. The reaction was stopped by the addition of 2 ml of chloroform:methanol (1:1). After thorough mixing, the phases were separated by centrifugation and the lower organic phase was removed. The aqueous phase together with the insoluble interface material was re-extracted with 1 ml chloroform and after phase separation, the lower phase was removed and combined with the original organic phase. Non lipid material was removed from the organic phase by washing with an equal volume of chloroform:methanol:water (3:48:47). After centrifugation the organic phase was

transferred to a scintillation vial, evaporated to dryness and assayed for radioactivity after adding 5 ml scintillation cocktail using liquid scintillation counter Beckman L.S. 1801.

3.2.16 Other Methods

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Chapter 4

4.0 RESULTS

4.1 ISOLATION AND PURIFICATION OF DIFFERENT SUBCELLULAR MEMBRANE FRACTIONS

Fig. 4.1 is the schematic presentation of the procedure followed for the isolation and purification of subcellular membrane fractions from peanut cotyledons. Since homogenization was carried out in an isotonic solution, the integrity of the plant membranes was largely preserved. Inclusion of EDTA in the homogenization medium introduced density variation in plasma membrane (PM) and rough endoplasmic reticulum (RER) by causing detachment of ribosomes from the latter. The smooth endoplasmic reticulum (SER), produced from RER, had lower density than PM and thus could be separated easily.

The first step in the purification scheme was the removal of heavy organelles such as nuclei, plastids, mitochondria, cell walls and other microbodies along with unbroken cells by centrifugation at 12,000 xg for 20 min. The supernatant fluid, that is, the post-mitochondrial fraction, on further centrifugation at 135,000 xg for 60 min resulted in a pellet and a clear light yellowish fluid (cytosol). The pellet represented the crude microsomes, mainly consisting of the PM, SER and Golgi apparatus (GA).

The PM fraction was separated from the SER and GA by centrifugation on a two step density gradient consisting of 34/20% sucrose solution in the buffer (Step III and IV). After centrifugation at 80,000 xg for 90 min, the PM fraction settled down as a

pellet at the bottom of the centrifuge tube, whereas the GA and SER formed a discrete band at the interface of 34/20% sucrose gradient. Final purification of PM fraction (Step V and VI) was achieved by using a six-step density gradient consisting of 45%, 38%, 34%, 30%, 25% and 20% sucrose. After centrifugation at 95,000 xg for 120 min a sharp band was formed at the 45/38% interface, corresponding to sucrose density of about 1.17g/cm^3. This fraction exhibited about 12-fold enrichment over the crude microsomes as adjudged by the specific activity of the PM marker enzyme, 1,3-β-D-glucan synthase (Table 4.1).

The band at interface of 34/20% of sucrose gradient (Step IV), containing GA and the SER was removed carefully with the help of Pasteur pipette, diluted with 25 mM Tris-HCl buffer, pH 7.2, to adjust the sucrose concentration to 10% and pelleted by centrifugation at 135,000 xg for 60 min. This fraction was further subjected to a three-step sucrose density gradient (43%, 37% and 25%) centrifugation for 180 min at 135,000 xg. A major band was formed at the interface of 37/25% sucrose gradient. The band corresponded to a density of 1.15g/cm^3. The inosine diphosphatase (IDPase), marker enzyme for GA, was greatly enriched in this fraction. This marker enzyme showed about 10-fold higher activity in the GA fraction as compared to its activity in PM or the SER. In addition, the activity of 1, 3-β-D-glucan synthase and mannosyltransferase, which are the marker enzymes for PM and SER, was insignificant indicating that the membrane fraction at the 37/25% interface contained largely GA and was practically free from cross contamination of PM and SER (Table 4.1). As judged by the specific activity of the marker enzyme, the GA fraction was enriched by about 4-fold. This fraction was used as GA membrane fraction.

The supernatant fraction above the 37/25% sucrose gradient interface band, corresponding to a density of 1.1 g/cm^3, was found to be highly rich in the dolichol

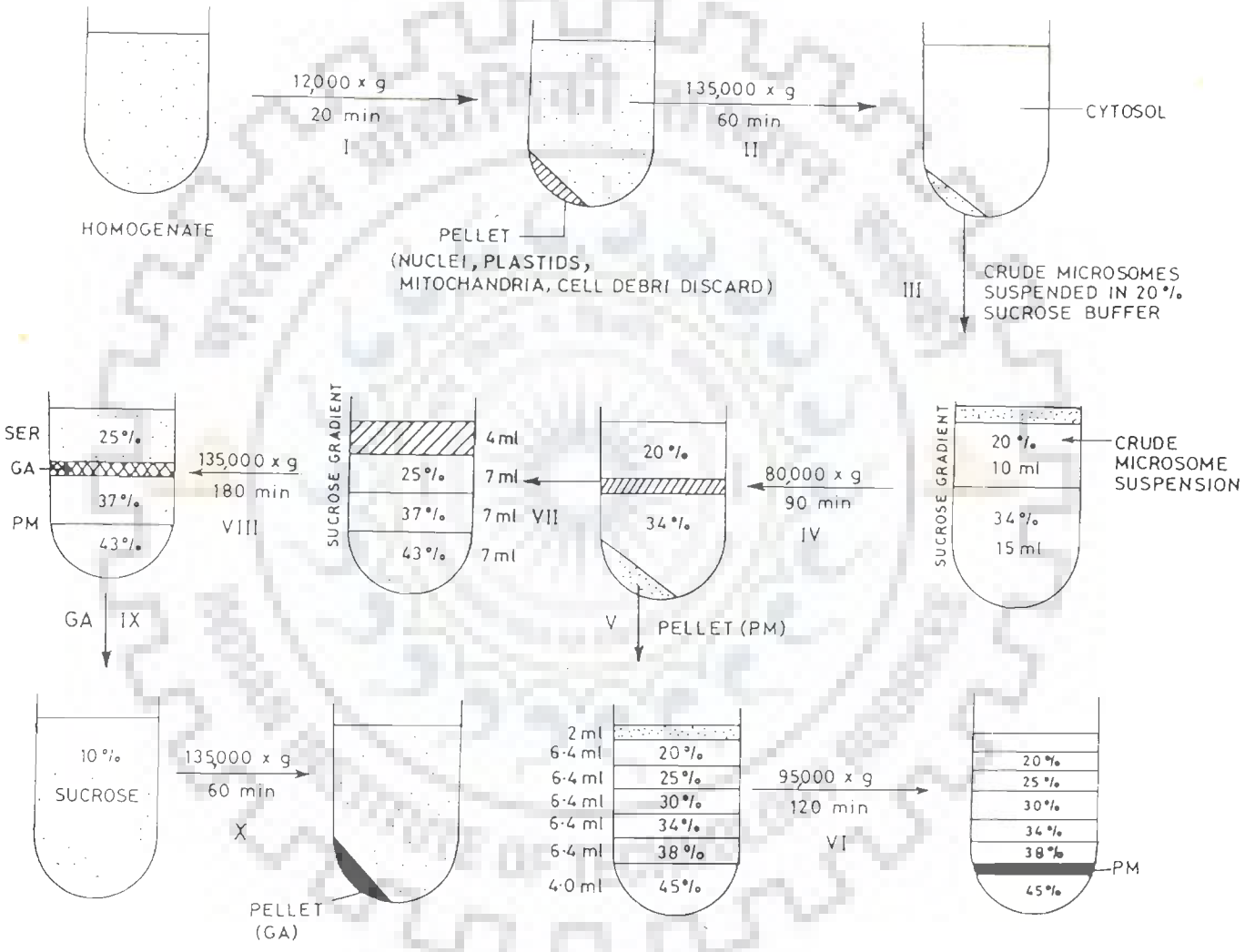


Fig.4.1 SCHEME FOR ISOLATION OF SUBCELLULAR FRACTIONS

TABLE 4.1 DISTRIBUTION OF MARKER ENZYMES IN DIFFERENT SUBCELLULAR FRACTIONS OF THE COTYLEDONS OF 7-DAYS OLD GERMINATING PEANUT SEEDLINGS

Fraction	Activity of Marker Enzyme		
	1,3-β-D-Glucan Synthase (cpm/mg-protein)	IDPase[a] (μ mole Pi/mg-protein)	Mannosyltransferase[b] (cpm/mg-protein)
Crude Microsomes	20620 ± 2500	23.8 ± 5.2	61288 ± 3800
Plasma Membrane (density 1.17 g/cm^3)[c]	256690 ± 4500	8.2 ± 2.7	3080 ± 230
Golgi Apparatus (density 1.15 g/cm^3)[c]	3625 ± 500	83.7 ± 9.1	18661 ± 900
Smooth Endoplasmic Reticulum (density 1.10g/cm^3)[c]	3224 ± 400	9.8 ± 3.4	531320 ± 6000

a. IDPase : Inosine Diphosphatase
b. Values obtained with 1μM dolichol phosphate as exogenous acceptor of mannose from GDP-[^{14}C] mannose
c. Represents the sucrose gradient at which the band of a specific membrane fraction is formed

phosphate : GDP-mannose-mannosyltransferase (MTase) activity with little activity of either 1,3-β-D-glucan synthase or IDPase, the marker enzymes for PM and GA. On the basis of the specific activity of SER marker enzyme, MTase, approximately 8.5-fold purification of SER fraction was achieved relative to the crude microsomes (Table 4.1). This fraction was also almost free from cross contamination of PM and GA membrane fractions as indicated by the extremely low activity of 1,3-β-D-glucan synthase and IDPase which are the marker enzymes for PM and GA.

In summary, it can be assumed that the scheme followed for the separation of various subcellular membrane fractions from the peanut cotyledons resulted in substantially pure PM, GA and SER fractions which were free from cross contamination.

4.2 SUBCELLULAR DISTRIBUTION OF AMPase IN GERMINATING PEANUT COTYLEDONS

The results of the subcellular distribution of AMPase in cotyledons of 7-days old germinating peanut seedlings are summarized in Table 4.2. The post-mitochondrial fluid (12,000 xg supernatant), which was used to prepare the crude microsomal fraction, contained about 1350 units (one unit being the amount of enzyme protein that would release one μ mole Pi/min from 5'-AMP under standard assay conditions) of AMPase activity per gram fresh weight of peanut cotyledons. About 6% of the total activity was found to be associated with the crude microsomal fraction (12,000-135,000 xg pellet) containing largely PM, GA and the SER. The PM and combined GA and SER fractions accounted respectively, for about 4% and 2% of the total AMPase activity in the post-mitochondrial fraction. Of the total AMPase activity in the crude microsomal pellet, the PM and the combined GA and SER fractions accounted for approximately two-third and one-third activity, respectively. Whether the AMPase forms in the PM, GA and SER are same, modified or represent altogether

TABLE 4.2 SUBCELLULAR DISTRIBUTION OF AMPase IN DIFFERENT FRACTIONS OF COTYLEDONS OF 7-DAYS OLD GERMINATING PEANUT SEEDLINGS

Fraction	Total Protein (mg/g fresh tissue)	Total AMPase Activity Per Gram Fresh Tissue (μ mole Pi/min)	Specific Activity (μ mole Pi/min/ mg-protein)	Recovery (%)
Post-Mitochondrial Fluid (12,000 xg supernatant)	24.10	1040.85	43.19	100.00
Crude Microsomal Pellet (12,000-105,000 xg)	2.7	72.77	26.97	6.23
Plasma Membrane (density 1.15g/cm^3)	0.78	47.82	61.30	4.59
GA + ER (density 1.1-1.15g/cm^3)	1.24	20.82	16.36	1.95

different forms of the enzymes, is not known at the moment. However, in the present study an attempt has been made to clarify this point by comparing the properties of the purified enzymes from the PM and the GA fractions.

4.3 PURIFICATION OF THE PM-AMPase

Table 4.3 summarizes the results obtained at each step during the purification of AMPase from PM. The separation of PM from the crude microsomal membrane fraction (12,000-135,000 xg pellet) resulted in approximately 2.6-fold purification relative to the crude fraction, with a yield of 67%. The solubilization of the membrane-bound enzyme by CHAPS extraction further increased the purification to about 5.2-fold. The yield at this stage was about 45% with respect to the crude membrane fraction. However, if the PM is taken as starting material, the yield would be about 66%. The next step of purification involved gel filtration chromatography on Sephadex G-150. The solubilized enzyme was dialyzed overnight against 100 volumes of 10mM Tris-HCl buffer, pH 7.2, with 0.04% β-mercaptoethanol, before loading onto the column, previously equilibrated with the same buffer. The column was washed with equilibration buffer and fractions were analyzed both for protein content and AMPase activity. The elution profiles of the AMPase activity and the protein are shown in Fig. 4.2. The enzyme (AMPase) activity was eluted in a single sharp peak comprising of fraction numbers from 60 to 68. It can be seen that the enzyme peak is fully overlapping the protein peak eluting in these fractions. Top three fractions, viz. fraction numbers 63, 64 and 65, were pooled and analyzed for the AMPase activity and protein content. In this step a purification of about 5-fold was achieved. The overall purification and yield after gel filtration step was about 27-fold and 22%, respectively (Table 4.3). A large quantity of protein was eluted in fractions preceding and succeeding the enzyme peak, indicating that gel filtration was a very effective purification step. Further, the enzyme was stable and active.

TABLE 4.3 PURIFICATION OF AMPase FROM THE PLASMA MEMBRANE FRACTION OF COTYLEDONS OF 7- DAYS OLD GERMINATING PEANUT SEEDLINGS

Fraction[a]	Total Protein (mg)	Total AMPase Activity	AMPase Specific Activity (μ mole Pi/min)	Purification (fold) (μ mole Pi/min/ mg-protein)	Yield (%)
Crude Microsomes (12,000-135,000 xg pellet)	726.5	17441.6	24.01	-	100.00
Plasma Membrane	189.1	11751.9	62.13	2.58	67.38
CHAPS[b] Solubilized (135,000 xg super-natant fluid)	62.1	7799.9	125.56	5.22	44.72
Gel Filtration (x5)[c] (Sephadex G-150)	6.6	3890.0	589.40	27.00	22.30
DEAE-Cellulose (x5)[c]	1.8	137.2	76.22	3.17	0.8

a. Various fractions represent 200g, fresh weight of cotyledons of 7-days old germinating peanut seedlings
b. (3-[(3-Cholamidopropyl) - dimethylammonio] -1- propane-sulfonate)
c. Values are from five runs

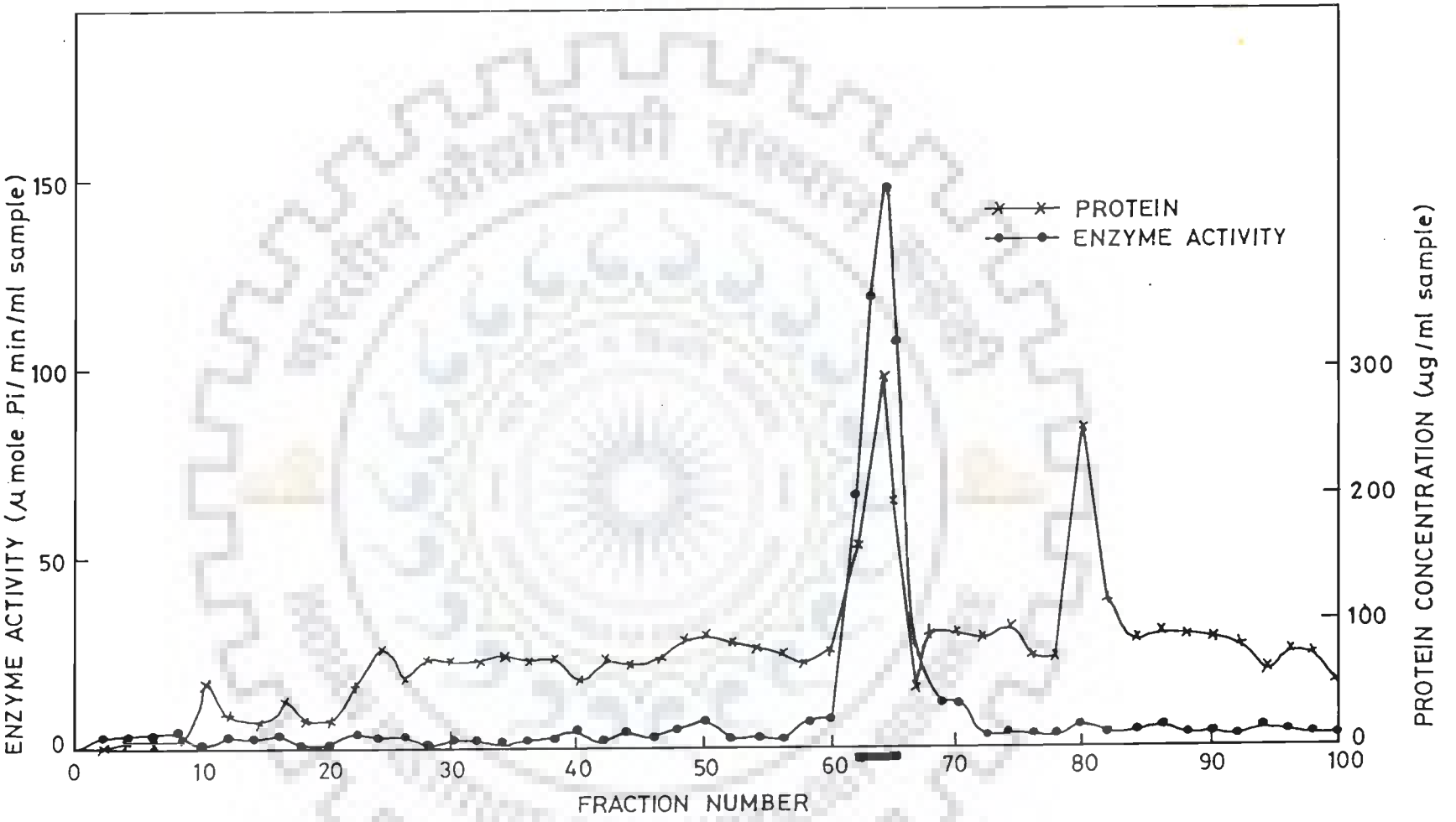


Fig.4.2 PURIFICATION OF PLASMA MEMBRANE AMPase ON SEPHADEX G-150 COLUMN
5 ml (ca12.5 mg) CHAPS solubilized protein was dialyzed for 6h against 10 mM Tris - HCl, pH 7.2, with 0.04% β-mercaptoethanol and loaded on Sephadex G - 150 column. Fractions of 2 ml each were collected at flow rate of 1 ml /20 min. The protein content and enzyme activity were determined as described in Material and Methods. The enzyme was eluted in one major peak (63-65) (—).

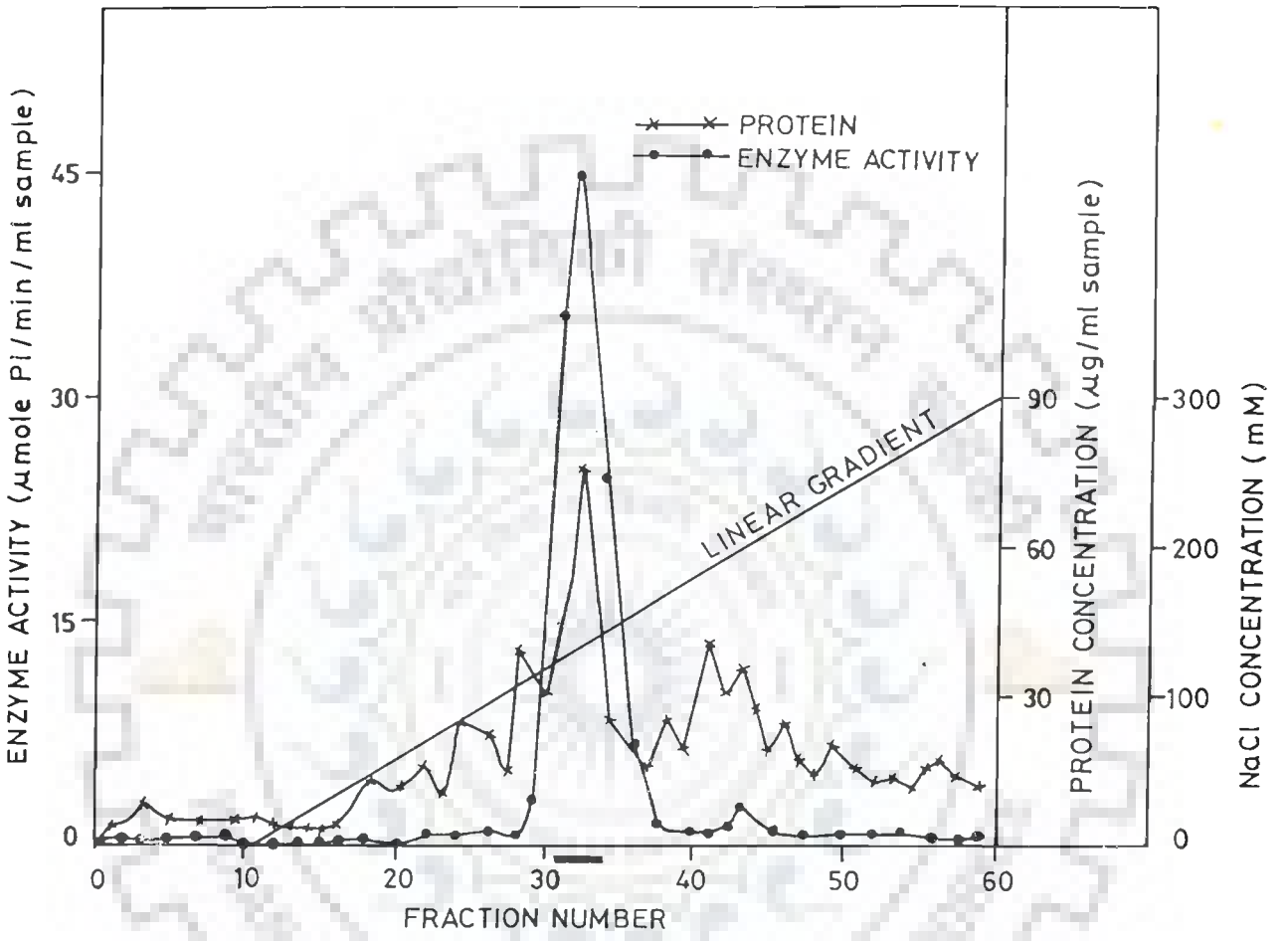


Fig.4.3 PURIFICATION PROFILE OF PLASMA MEMBRANE AMPase FROM DEAE - CELLULOSE COLUMN

The peak fractions (63 to 65) obtained after gel filtration chromatography were loaded on DEAE - cellulose column (1x1.5 cm). The absorbed proteins were eluted by a linear gradient of NaCl (0-300mM) in 10mM Tris - HCl (pH 7.2) with 0.04% β-mercaptoethanol and 20% glycerol. Fractions of 2ml each were collected at flow rate of 0.2 ml/min and were analysed for protein content and enzyme activity. The elution profile shows enzyme activity obtained at 110 mM NaCl concentration. The major peak (—) constituted fractions from 21 to 24.

The final step of purification was the ion-exchange chromatography on a DEAE-cellulose column. The absorbed proteins were eluted with a linear gradient of NaCl (0-300mM) in 10mM Tris-HCl buffer, pH 7.2, containing 20% glycerol, 0.01% CHAPS and 0.04% β-mercaptoethanol. The presence of glycerol in the buffer was needed for the stability of enzyme, which was otherwise extremely labile during this step of purification. Fig. 4.3 shows the elution profiles of the AMPase activity and protein content. The AMPase activity was eluted as a single symmetrical peak at approximately 130mM NaCl gradient concentration, corresponding to 30 to 35 fraction numbers. Top three fractions of the peak, i.e. fraction numbers 31, 32 and 33, were pooled and AMPase activity was determined. Surprisingly, the specific activity of the enzyme decreased nearly 8 times with an apparent purification decreasing to 3-fold from 27-fold (Table 4.3) although, as evident from the protein elution profile (Fig. 4.3), substantial amount of proteins without AMPase activity were separated from the enzyme by the DEAE-cellulose column chromatography. These results indicate that inactivation of the enzyme was probably due to extremely labile nature of the purified enzyme obtained after the DEAE-cellulose column. Nevertheless, the enzyme was still sufficiently active, comparable with the specific activity of the PM-bound enzyme and was immediately used for further studies, unless stated otherwise.

4.4 HOMOGENEITY AND MOLECULAR MASS OF THE PURIFIED AMPase

The homogeneity of the purified enzyme obtained from the DEAE-cellulose column was tested by SDS-PAGE followed by silver staining of protein bands and also by Western blotting and double immunodiffusion techniques using polyclonal antibodies raised against the purified enzyme as described under Material and Methods. Fig. 4.4 shows the SDS-PAGE protein profiles of the purified enzyme from DEAE-cellulose column (lane A); CHAPS-solubilized enzyme fraction (lane B) and standard molecular weight marker proteins (lane C). The purified protein migrated on

the gel, under fully-dissociated conditions, as a tight single protein band indicating that the enzyme preparation was homogeneous and contained no contaminating proteins whereas the CHAPS solubilized enzyme fraction resolved in a large number of protein bands. From migration pattern on the SDS-PAGE it was apparent that the enzyme is of relatively low molecular mass. Since, SDS-PAGE was performed under fully-dissociated conditions, presence of the single protein band suggests that the AMPase contains only a single polypeptide chain. However, possibility of the presence of more than one polypeptide chain of exactly same molecular size can not be ruled out.

Fig. 4.5 shows the photograph of double immunodiffusion patterns of the cross reactivity of antibodies prepared against the purified enzyme and that of the preimmune serum with purified AMPase. It can be seen that AMPase antibodies cross reacted with the enzyme giving a single sharp band of AMPase antibody complex, while no AMPase-antibody band was detected where preimmune serum was used in place of the antiserum. The sharpness of the AMPase - antibody band was a clear indication of the homogeneity of the AMPase.

The homogeneity of the purified AMPase was further confirmed by Western blotting (Fig. 4.6). It was found that the polyclonal antibodies were highly specific for the AMPase as shown on the Western blot. Only one protein-antibody complex band was identified in the crude CHAPS-solubilized PM protein extract containing AMPase and partially purified AMPase from Sephadex G-150 column, which was exactly identical with that obtained with the purified AMPase from DEAE-cellulose column. The lane of the extreme right (lane E) shows the positive control protein for the detection of antigen by the antibodies, indicating the detection efficiency of the Western blotting. Hence the results clearly show that AMPase was purified to homogeneity using normally employed conventional method for the purification of membrane proteins.

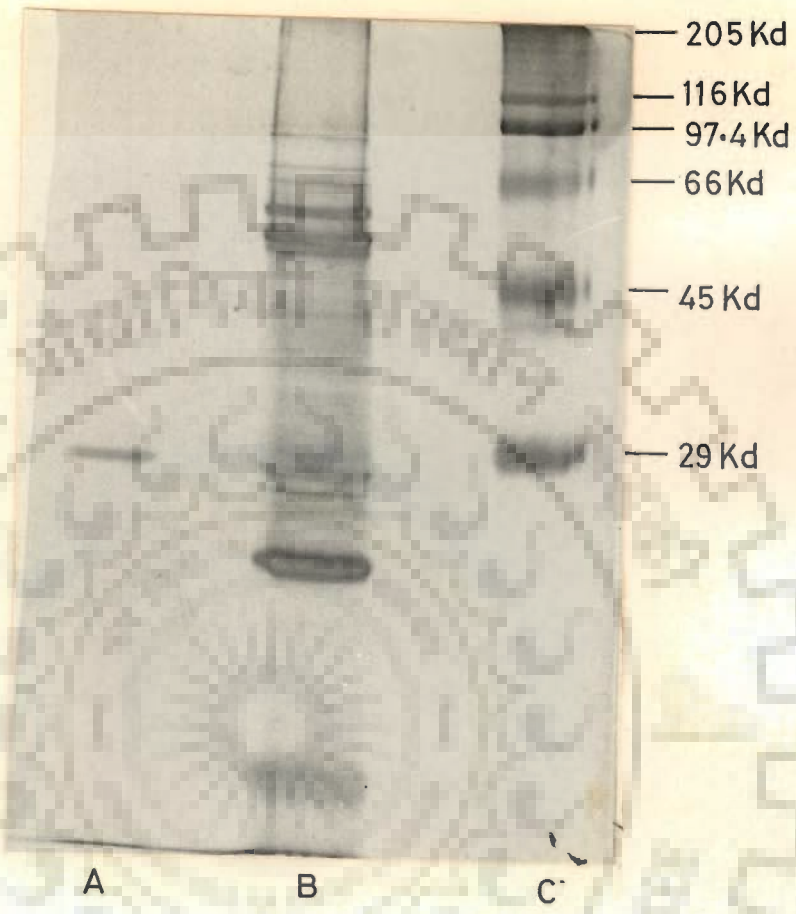


Fig.4.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SILVER STAINING)
SDS- Polyacrylamide gel electrophoresis was carried out as described in Material and Methods to check the homogeneity of purified AMPase. Gel was silver stained by method of Davis et al. (1986). Photograph shows

A purified AMPase

B plasma membrane enzyme solubilized by CHAPS

C standard molecular weight markers as:
29 Kd, Carbonic anhydrase; 45 Kd, Egg albumin;
66 Kd, Bovine albumin, ; 97.4 Kd, Phosphorylase B;
116 Kd; β-Galactosidase; 205 Kd, Myosin.

Fig.4.5 IMMUNODIFFUSION PATTERNS OF ANTISERUM AND PREIMMUNE SERUM

The conditions used for immunodiffusion were those as described under Material and Methods. As seen in the photograph the different wells contained the following:

Central Well	:	purified AMPase
Well 1	:	preimmune serum in 1:5 dilution in PBS-azide
Well 2	:	preimmune serum in 1:10 dilution in PBS-azide
Well 3	:	antiserum in 1:5 dilution in PBS-azide
Well 4	:	antiserum in 1:10 dilution in PBS-azide.

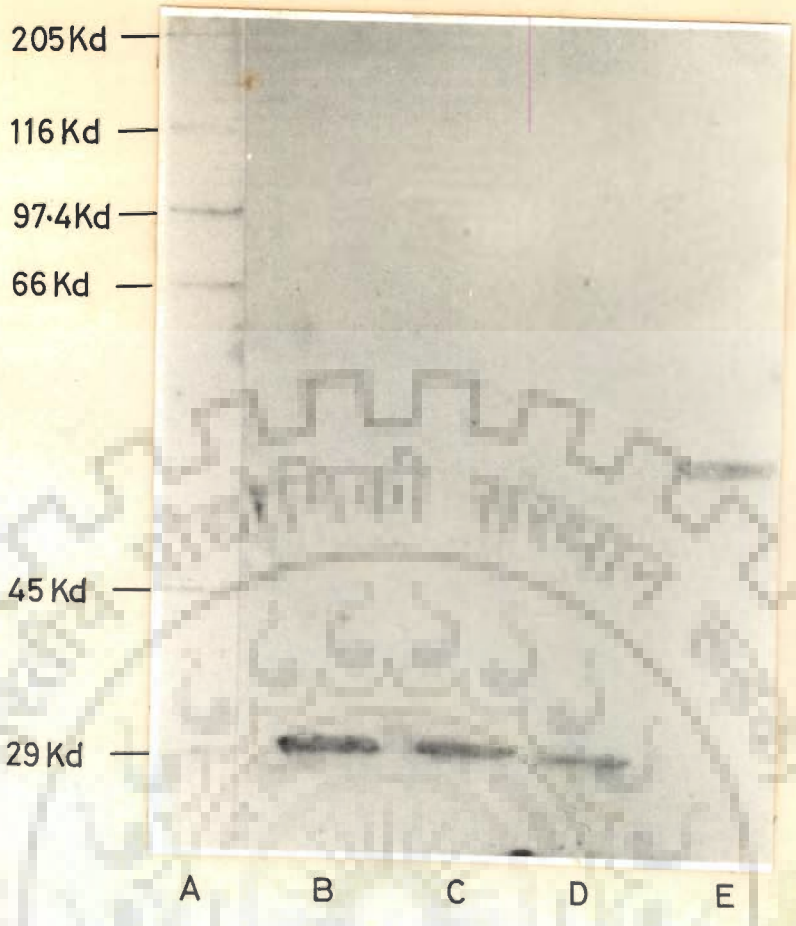


Fig.4.6 WESTERN BLOTTING EXPERIMENT WITH ENZYME AT VARIOUS STAGES OF PURIFICATION

SDS-Polyacrylamide gel electrophoresis was carried out as described in Material and Methods with the different fractionation stages of purification along with positive protein and molecular weight marker. Proteins were transferred to nitrocellulose sheet using semi-dry transfer apparatus as described in Material and Methods and then the nitrocellulose sheet was cut from the side of molecular weight marker to check the transfer. Rest of proteins were subjected to Western blotting using Amersham kit. Photograph shows

A : Standard molecular weight markers as:
29 Kd, Carbonic anhydrase; 45 Kd, Egg albumin;
66 Kd, Bovine albumin, ; 97.4 Kd, Phosphorylase B;
116 Kd; β-Galactosidase; 205 Kd, Myosin.

B : Solubilized enzyme

C : Enzyme eluted after Sephadex G-150 column

D : Purified enzyme

E : Positive control protein

4.5 CHARACTERIZATION OF THE PM-AMPase

4.5.1 Molecular Mass Of The Purified PM-AMPase

The molecular mass (M_r) of the purified PM-AMPase was determined by SDS-PAGE under fully-reducing conditions using protein standards of known molecular masses from Sigma Chemical Co. The calibration curve, $\log_{10} M_r$ versus relative migration of protein standards with respect to bromophenol blue, used for the computation of molecular mass is shown in Fig. 4.7. The M_r of the purified PM-AMPase was found to be 26,300 daltons. This enzyme is, therefore, a low molecular mass species. However, it is quite possible that the purified AMPase is a monomeric form of the native enzyme, which is a homo-oligomer consisting of two or more subunits of the same polypeptide monomer.

4.5.2 Optimum pH Of The PM-AMPase

The pH dependence of the purified peanut PM-AMPase activity (Fig. 4.8) shows the pH optimum between pH 5.0 and 5.5. The enzyme activity below and above this pH range sharply declined. For instance, at pH 4.5 and 6.0 the enzyme activity was only about 80% and 45%, respectively of the maximum activity measured at the optimum pH 5.0. In all experiments described in the thesis, the PM-AMPase activity was, therefore, assayed at pH 5.0, unless stated otherwise.

4.5.3 Kinetic Parameters

The K_m and V_{max} values of the PM-AMPase for 5'-AMP as substrate, as determined by LineWeaver - Burk Plot (Fig. 4.9) were found to be 0.58mM and 5.9 µ mole Pi/min/mg-protein, respectively. At higher substrate concentration, slight inhibition of the enzyme activity was noticed.

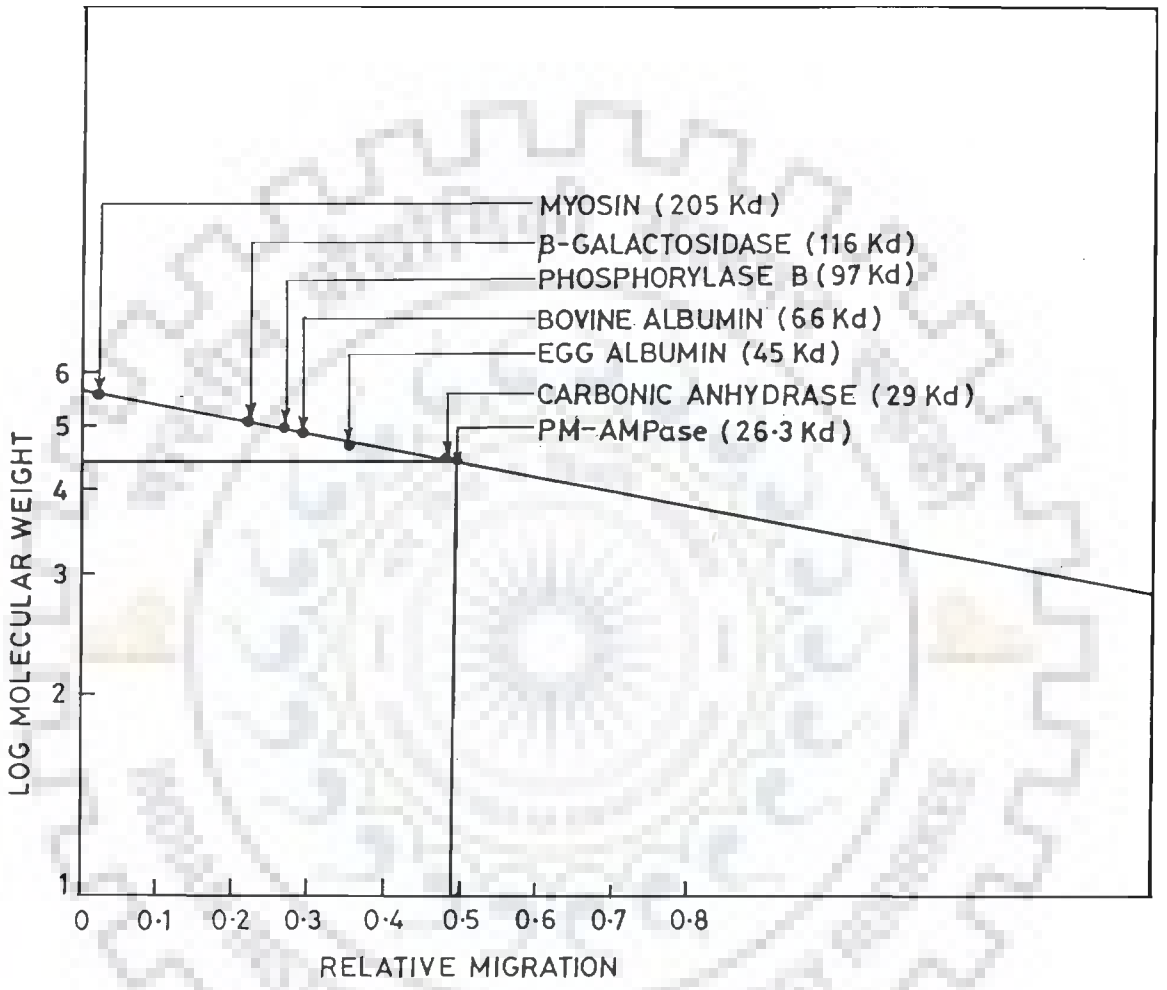


Fig.4.7 ESTIMATION OF MOLECULAR WEIGHT OF PURIFIED PM-AMPase.
The relative migration of standard molecular weight markers is plotted against log of molecular weight to calculate the molecular weight of purified PM-AMPase. As derived from this plot the molecular weight of purified PM-AMPase is found to be 26.3Kd.

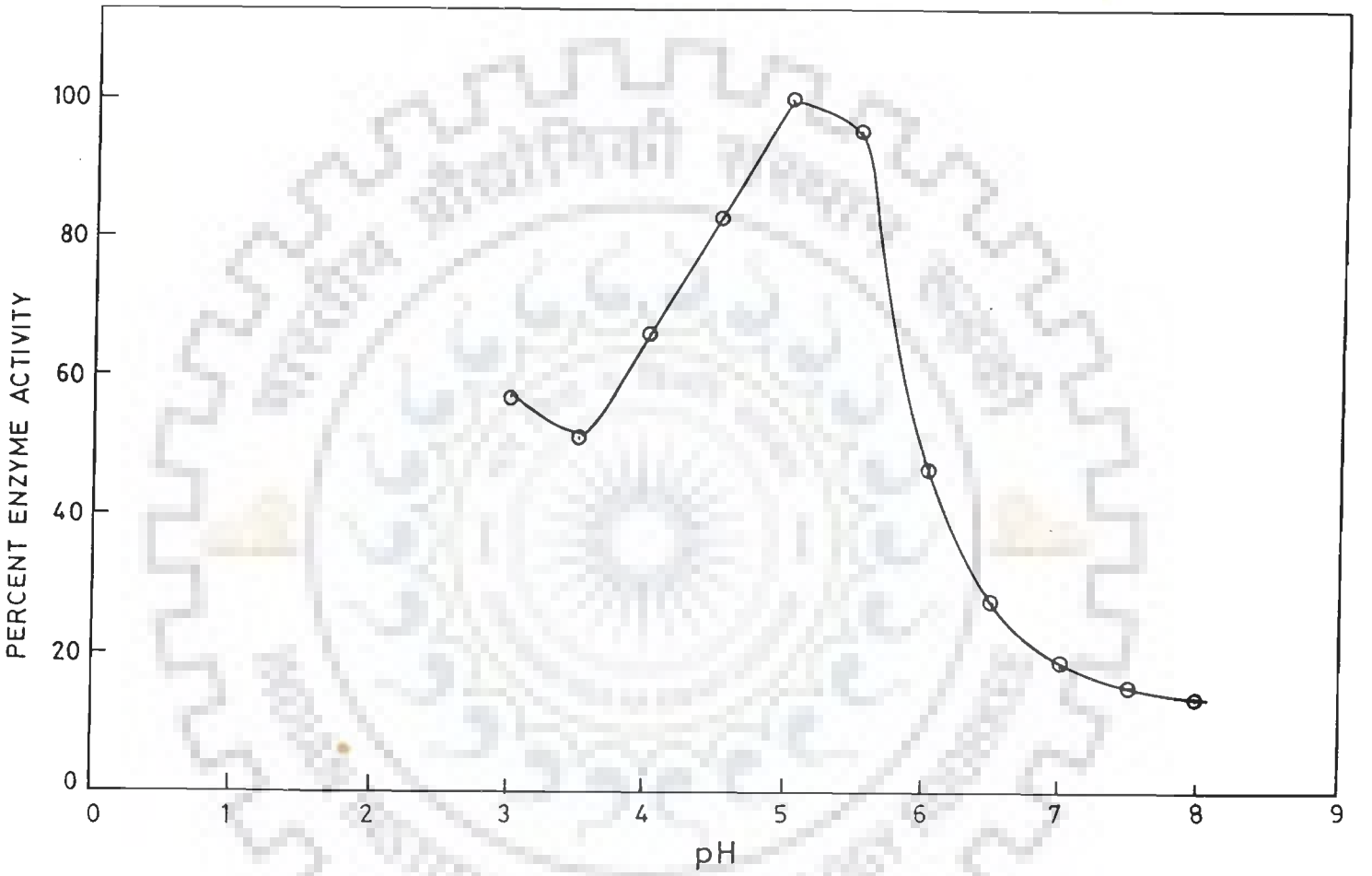


Fig.4.8 **pH OPTIMA OF PURIFIED PM-AMPase**
The enzyme activity was measured in the standard assay conditions except that the buffer and pH were varied. Buffers used were 50 mM sodium-acetate (pH 3.5 to 5.5) and 50 mM Tris-HCl (pH 6.0 to 8.0). Maximum enzyme activity was taken as 100%.

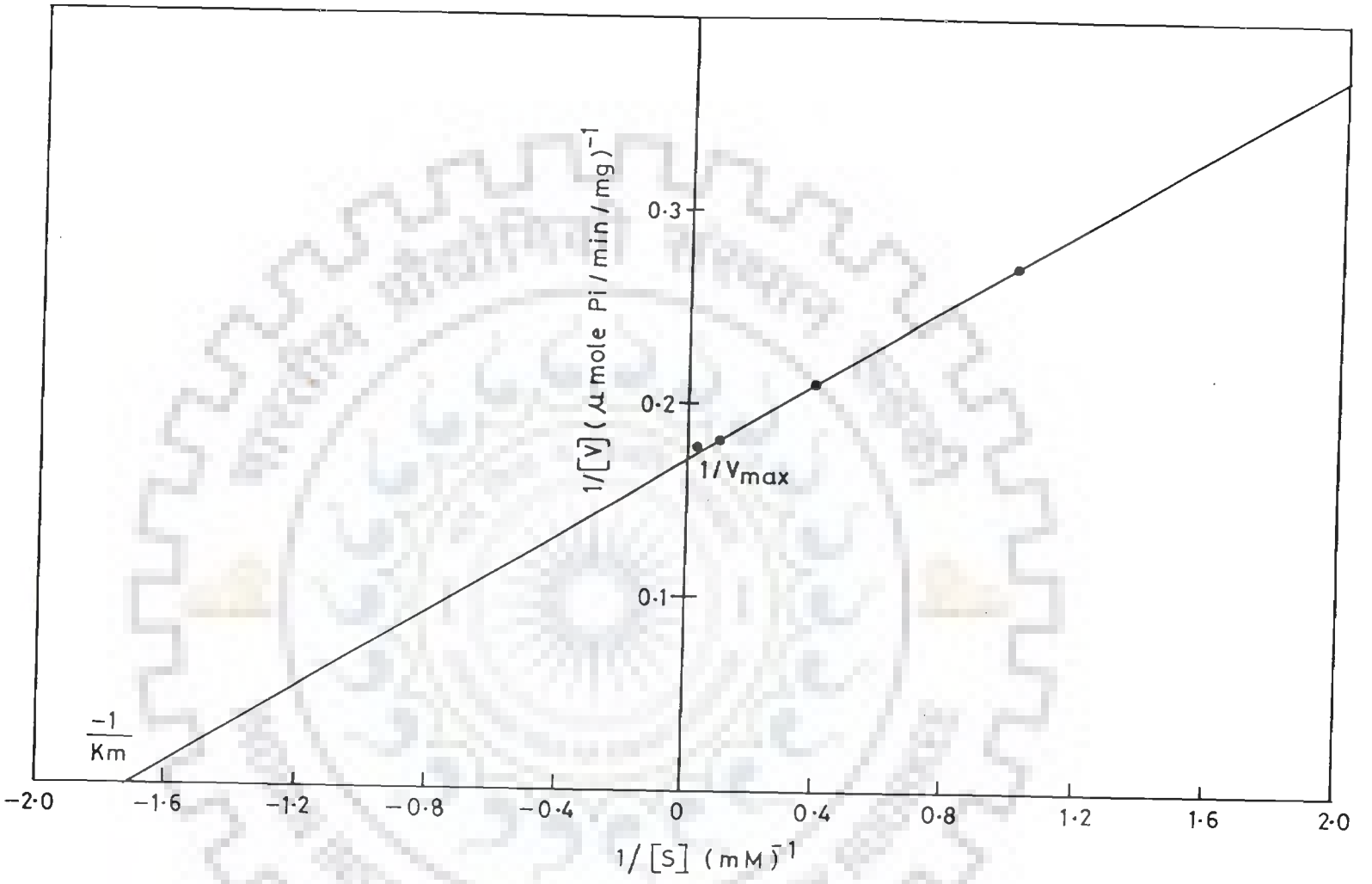


Fig.4.9 LINEWEAVER-BURK PLOT OF PURIFIED PM-AMPase

This is the Lineweaver-Burk plot of purified PM-AMPase using 5'-AMP as substrate in 50mM sodium-acetate buffer, pH 5.0. Enzyme was assayed at different concentrations of substrate under standard assay conditions. K_m and V_{max} values were computed from the intercepts on X-axis and Y-axis, respectively.

4.5.4 Substrate Specificity

The substrates tested for the hydrolysis by the purified PM-AMPase and the membrane-bound PM-AMPase were 5'-AMP, 5'-GMP, 5'-UMP, 5'-ADP, 5'-GDP, 5'-UDP, 5'-ATP, glucose-1-phosphate, glucose-6-phosphate and p-nitrophenyl phosphate at 2.5mM concentration. The results summarized in Table 4.4 clearly show that the purified PM-AMPase was highly specific for 5'-AMP. Other nucleoside phosphates and phosphorylated sugars were not significantly hydrolyzed. Even the 5'-ADP and 5'-ATP were hydrolyzed by the purified enzyme to only 20% and 15% of the rate of hydrolysis 5'-AMP, indicating that 5'-ADP and 5'-ATP were extremely poor substrates for the purified PM-AMPase. Since the enzyme was found homogeneous by SDS-PAGE and Western blotting using antiserum, it appears unlikely that the hydrolysis of 5'-ADP and 5'-ATP was due to the contamination of some other non-specific acid phosphatases. In contrast, the highly enriched PM fraction contained acid phosphatases which strongly catalyzed the hydrolysis of other nucleotides, glucose-1-phosphate and p-nitrophenyl phosphate. In fact, the hydrolysis rates of 5'-GMP and p-nitrophenyl phosphate by the PM-bound (unpurified) enzyme preparation were nearly comparable with the rate of hydrolysis of 5'-AMP. It is interesting to note that the purified PM-AMPase did not exhibit any phosphatase activity towards the p-nitrophenyl phosphate, a standard synthetic substrate used for assaying acid phosphatases of broad specificity. These results clearly indicate that the purified PM-AMPase was almost free from other acid phosphatases and 5'-nucleotidases that were present in the PM fraction. The results also prove the efficiency of the purification procedure adopted in the present study.

4.5.5 Stability Of The Purified PM-AMPase

During the final step of purification of the PM-AMPase, the specific activity of the enzyme decreased by almost 8 times (Table 4.3). One of the several reasons

TABLE 4.4 SUBSTRATE SPECIFICITY OF PEANUT PM-AMPase

Substrate	PM-Bound Phosphatase Activity		Purified PM-AMPase Activity	
	(μ mole Pi/min/ mg-protein)	(%)	(μ mole Pi/min/ mg-protein)	(%)
Adenosine-5′-monophosphate	62.13	100	76.22	100
Adenosine-5′-diphosphate	23.52	37.87	15.24	20.00
Adenosine-5′-triphosphate	23.41	37.69	11.62	15.25
Guanosine-5′-monophosphate	61.33	98.72	2.17	2.85
Guanosine-5′-diphosphate	49.23	79.24	3.62	4.75
Uridine -5′-monophosphate	19.23	30.95	5.76	7.56
Uridine -5′-diphosphate	0.22	0.36	5.80	7.61
Glucose -1-phosphate	18.66	30.04	5.80	7.61
Glucose -6-phosphate	5.54	8.92	0.72	0.95
p-Nitrophenyl phosphate	50.95	82.01	0.72	0.95

for the loss of enzyme activity could be the highly labile nature of the enzyme. This aspect was investigated further. Results presented in Fig. 4.10 induced the case, since the purified PM-AMPase was rendered completely inactive within 24h when stored in 10mM Tris-HCl buffer (pH 7.2) containing 0.04% β-mercaptoethanol and 0.01% CHAPS at -20°C. However, this purified AMPase showed a much better stability when stored in the presence of 20% glycerol for 7-days in the same buffer (Fig. 4.10). For example, in the presence of 20% glycerol the purified enzyme was found to retain approximately 85%, 80% and 50% of the activity after 24, 48 and 72h respectively, when stored at -20°C, whereas in the absence of glycerol the total enzyme activity was lost within 24h at -20°C. Thus, presence of glycerol in the buffer greatly helped in enhancing the stability of the purified enzyme.

4.5.6 Effect Of Phospholipids On Restoration Of Enzyme Activity

Another possible reason for the impairment of the activity and stability of the purified PM-AMPase (from DEAE-cellulose column) could be the removal of some activator and stabilizing factor(s) such as phospholipids which were originally present in the detergent solubilized as well as partially purified PM-AMPase from Sephadex G-150 column during the final step of purification. This possibility was investigated by using a partially active PM-AMPase obtained after 6 days storage at -20°C, showing only 20-25% residual activity and pre-incubating it with specific phospholipids before assaying the enzyme activity by standard assay procedure. The results (Table 4.5) show that except phosphatidylinositol (PI) which had an inhibitory effect, all other phospholipids used, namely phosphatidylcholine, phosphatidylserine and phosphatidylglycerol, partially restored the enzyme activity. The most effective in restoring the enzyme activity was phosphatidylcholine followed by phosphatidylglycerol. Phosphatidylcholine was able to restore the PM-AMPase activity upto about 46%. These results clearly indicate the important role of

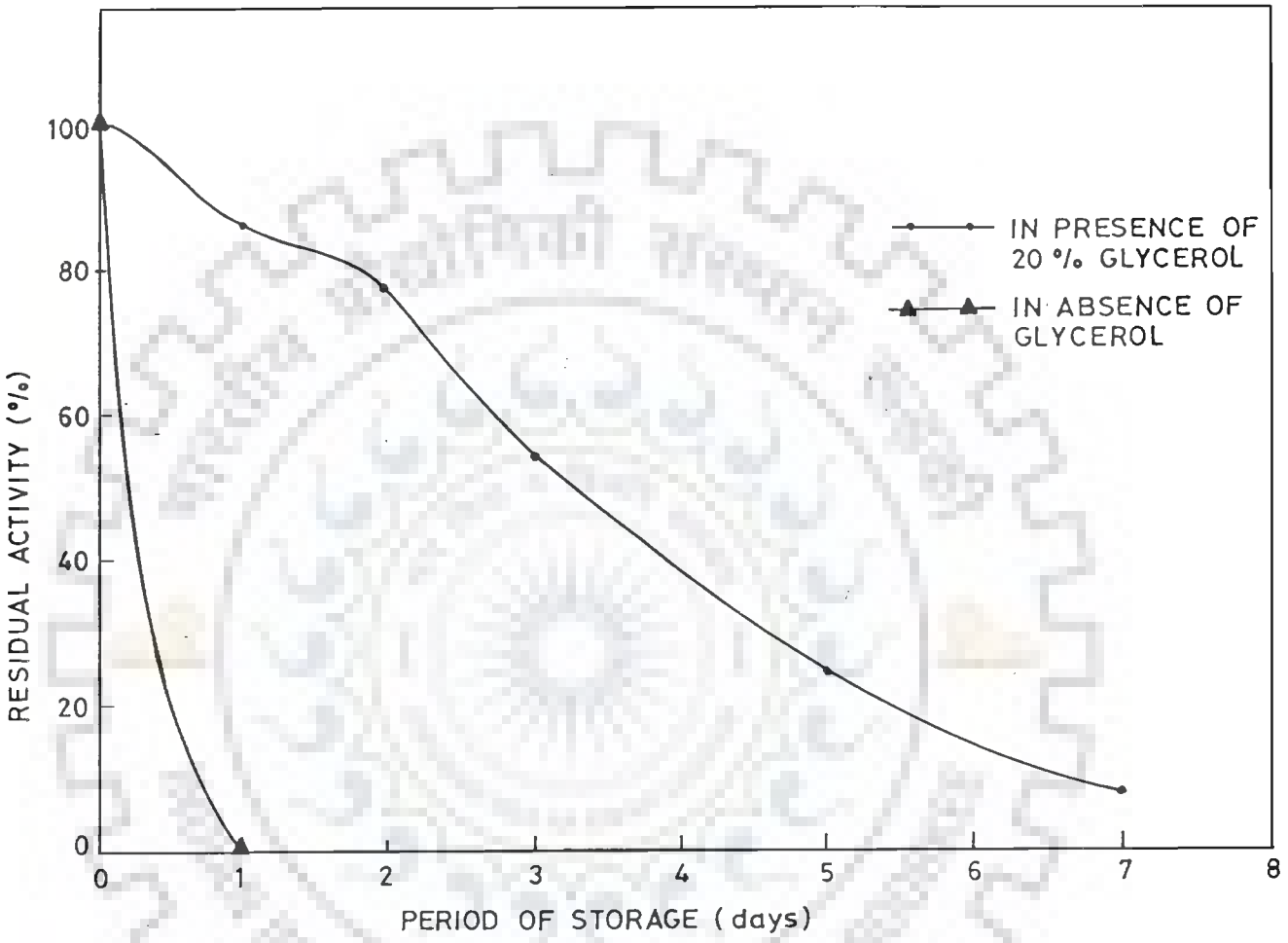


Fig.4.10 EFFECT OF PRESENCE OF GLYCEROL ON PURIFIED PM-AMPase

The stability of enzyme eluted after DEAE - cellulose column chromatography was studied. Figure shows the percent residual activity of purified enzyme in 10mM Tris-HCl (pH 7.2) with 0.04% β-mercaptoethanol, 0.01% CHAPS and 20% glycerol for 7-days and in the same buffer without glycerol for a period of 24h at -20° C.

TABLE 4.5 EFFECT OF DIFFERENT PHOSPHOLIPIDS ON THE RESTORATION OF THE ACTIVITY OF PARTIALLY ACTIVE PM-AMPase

Phospholipid Added	PM-AMPase[a] Specific Activity (μ mole Pi/min/ mg-protein)	Percent of Control
None	17.74	100
Phosphatidylcholine	25.88	145.88
Phosphatidylserine	19.82	111.72
Phosphatidylglycerol	23.76	133.93
Phosphatidylinositol	14.94	84.2

a. The PM-AMPase obtained from DEAE-cellulose column (specific activity 76.2 μ mole Pi/min/mg-protein) was stored in glycerol buffer at -20°C for 5-days. During the storage about 75% of the enzyme activity was lost. The partially active enzyme (specific activity 17.74 μ mole Pi/min/mg-protein) was pre-incubated with 2μg/ml phospholipid in ice for 15 min before assaying the activity by standard procedure. Control assays were performed concurrently without adding phospholipid in incubation mixture.

phospholipids in keeping the enzyme active and stable.

The restoration of the enzyme activity was further investigated by using an enzyme sample which was rendered 90% or more inactive by storage at -20°C in glycerol buffer for 7-days (Fig. 4.10) and by varying the concentrations of phosphatidylcholine. It was found that the restoration of enzyme activity by phosphatidylcholine was concentration dependent (Fig. 4.11). A nearly linear increase in the enzyme activity was observed upto a concentration of 6μg phosphatidylcholine per assay under standard assay conditions. After this, with the increase in concentration of phospholipid, the increase in enzyme activity was levelled off and at 8μg concentration of phosphatidylcholine per assay approximately 45% of the enzyme activity was restored. It would be, therefore, beneficial to use phosphatidylcholine in the elution buffer, especially in DEAE-cellulose column chromatography step which resulted in substantial loss of enzyme activity. These results also provide a possible explanation for the sharp decrease in the specific activity during the final step of purification of the enzyme.

4.5.7 Glycoprotein Nature Of PM-AMPase

The peanut cotyledon PM-AMPase was found to contain 37% (w/w) carbohydrate and appeared to be a glycoprotein. This conclusion is based on the following observations : (i) A single band on SDS-polyacrylamide gel corresponding to the PM-AMPase gave a positive reaction for periodate/Schiff's base staining for carbohydrate (Fig. 4.12) (ii) The digestion of the purified PM-AMPase with endo-H (endo-N-acetyl-β-D-glucosaminidase-H) released approximately 90% of the total carbohydrate from the enzyme in 24h (Fig. 4.13), indicating that the oligosaccharide moiety was N-linked high mannose type. About 10% of the carbohydrate was not released by endo-H digestion. Whether this represented the complex type structure

or O-glycosylated form, is not known (iii) Finally, the immunoprecipitation of the *in vitro* mannose - labelled glycoprotein showed a protein band which corresponded to the purified PM-AMPase (Fig 4.14).

4.5.8 Purified PM-AMPase Is Not A Metalloprotein

Peanut PM-AMPase, unlike the AMPase from animal tissues, is not a zinc containing protein as was found by the atomic absorption/emission spectroscopy of the purified enzyme (Fig. 4.15).

4.5.9 Immunological Properties Of PM-AMPase

In order to study the immunological properties of the PM-AMPase and to compare its immunological properties with those from other plant sources and sub-cellular fractions, antibodies against the purified enzyme were raised in NewZealand strain white female rabbits and purified on DEAE-cellulose column after $(NH_4)_2SO_4$ precipitation (45% saturation) as described by Mayer and Walker (1987). The preimmune serum was also subjected to the same treatment. Fig. 4.16 shows the elution profile of $(NH_4)_2SO_4$ precipitated protein fraction purified on DEAE-cellulose column. As expected, most of the protein, mainly IgG, eluted in the flow through volume. The peak fraction numbers 5 to 7 were pooled and precipitated with $(NH_4)_2SO_4$ as before. The precipitate was collected and dissolved in sodium phosphate buffer and stored in 0.5ml aliquots at -20°C.

Incubation of the antiserum made against the purified PM-AMPase with detergent solubilized enzyme resulted in immunoprecipitation of the PM-AMPase activity, as indicated by substantial decrease in the enzyme activity in the supernatant fraction obtained after removal of immune complex by centrifugation (Fig. 4.17). Lack of complete removal of the phosphatase activity from the detergent solubilized

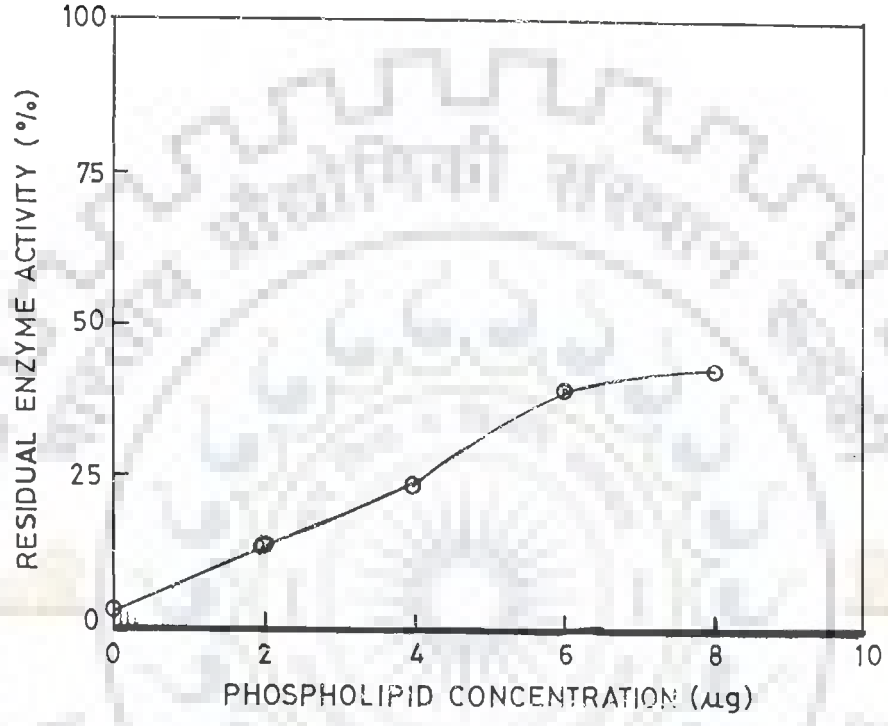


Fig.4.11 EFFECT OF PHOSPHATIDYLCHOLINE ON ACTIVITY OF PURIFIED PM-AMPase

After 7-days when the enzyme lost almost 90% of its activity, the effect of phosphatidylcholine was studied. Phospholipid was dried under nitrogen, 0.01% CHAPS and purified PM-AMPase was added to dried phospholipid and kept in ice for 15 min. Then the enzyme activity was assayed using standard protocol. Enzyme regained almost 45% activity in the presence of phosphatidylcholine.

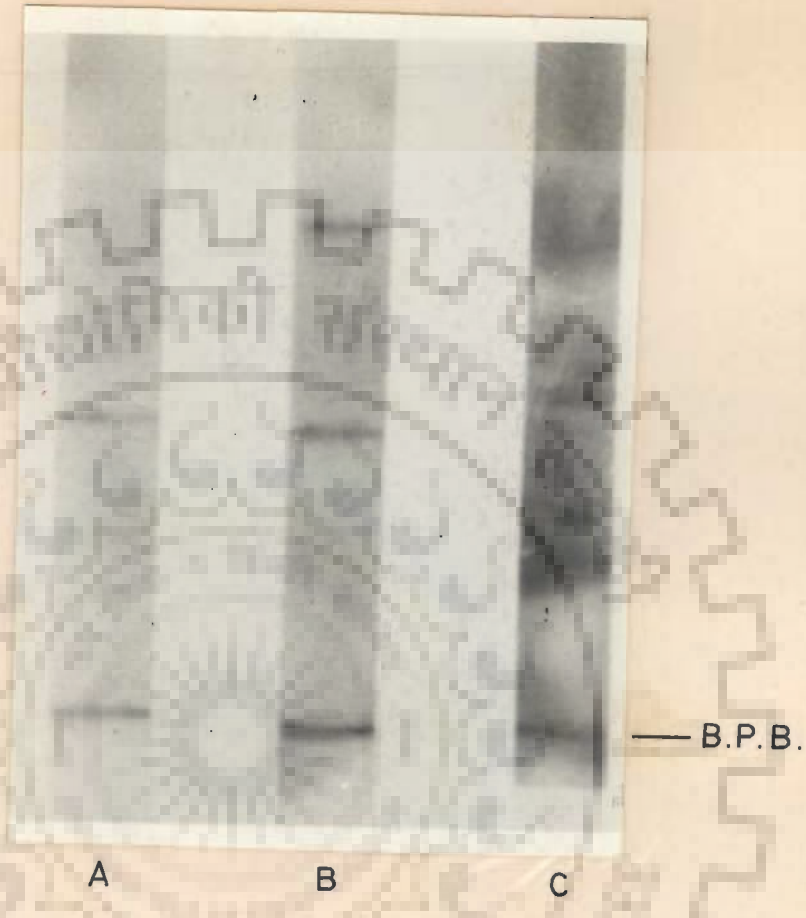


Fig.4.12 SCHIFF'S BASE STAINING OF PLASMA MEMBRANE AMPase.
SDS-Polyacrylamide gel electrophoresis was run as described in the Material and Methods and the gel was stained by Schiff's base. Photograph shows:
A : Purified PM-AMPase after DEAE-cellulose column
B : PM-AMPase after gel filtration chromatography
C : Microsomal membrane fraction

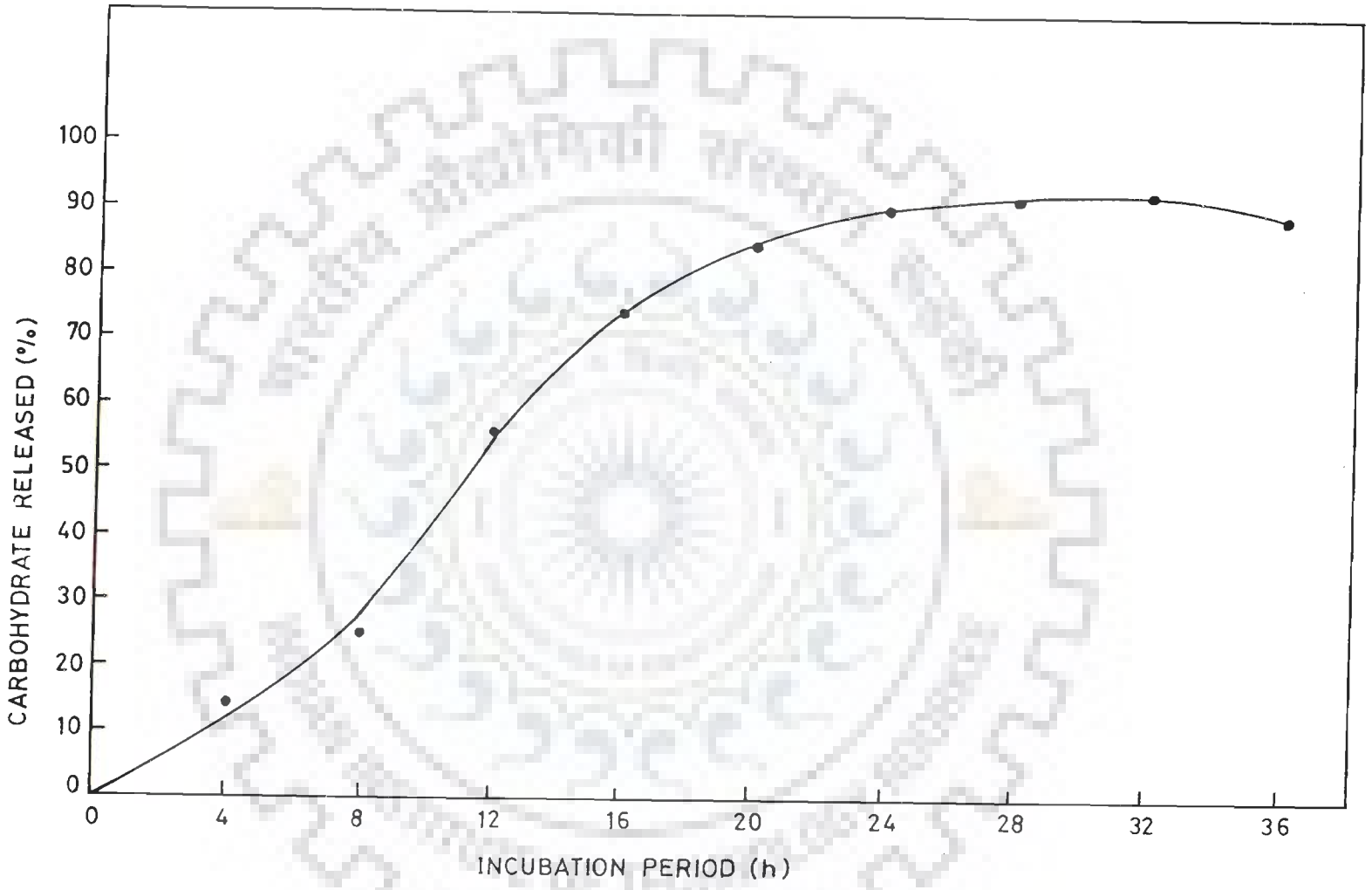


Fig.4.13 DEGLYCOSYLATION OF PM-AMPase BY Endo-H TREATMENT
The purified PM-AMPase was incubated with endo-H for varying time periods. After 24h almost 90% of the carbohydrates attached to the purified enzyme were released.

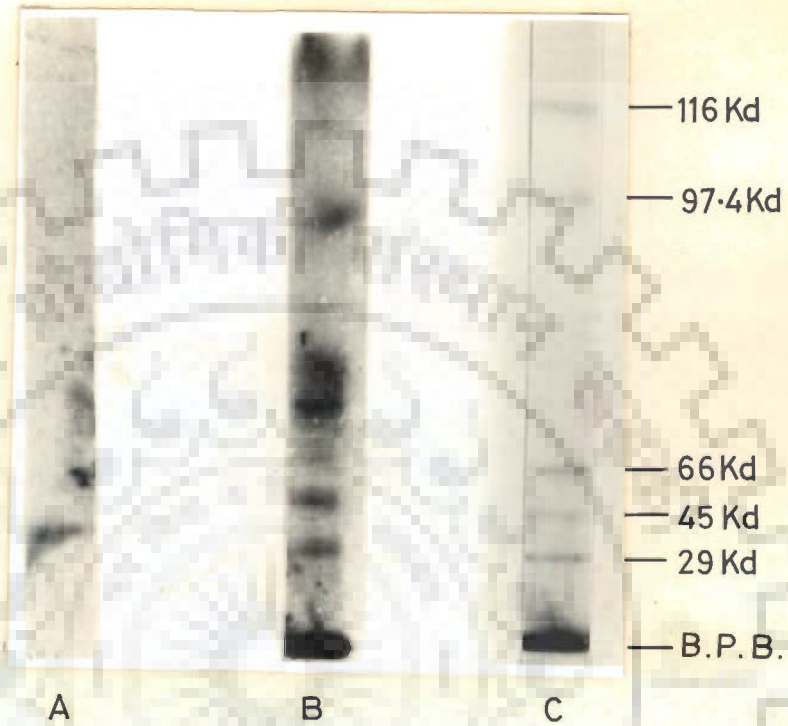


Fig.4.14 IMMUNOPRECIPITATION OF LABELLED GLYCOPROTEINS

[^{14}C] GDP-mannose labelled glycoprotein was precipitated with antibodies against purified PM-AMPase and SDS-Polyacrylamide gel electrophoresis of radioactive glycoprotein was carried out. Gel was treated with PPO and then dried at 70°C. The dried gel was kept for the autoradiography. The photograph shows X-ray film exposed for one month to the radiolabelled glycoprotein in the gel.

A : Glycoprotein precipitated with antibodies and protein A

B : Radiolabelled glycoprotein fraction

C : standard molecular weight markers as:
29 Kd, Carbonic anhydrase; 45 Kd, Egg albumin;
66 Kd, Bovine Albumin, ; 97.4 Kd, Phosphorylase B;
116 Kd; β-Galactosidase; 205 Kd, Myosin.

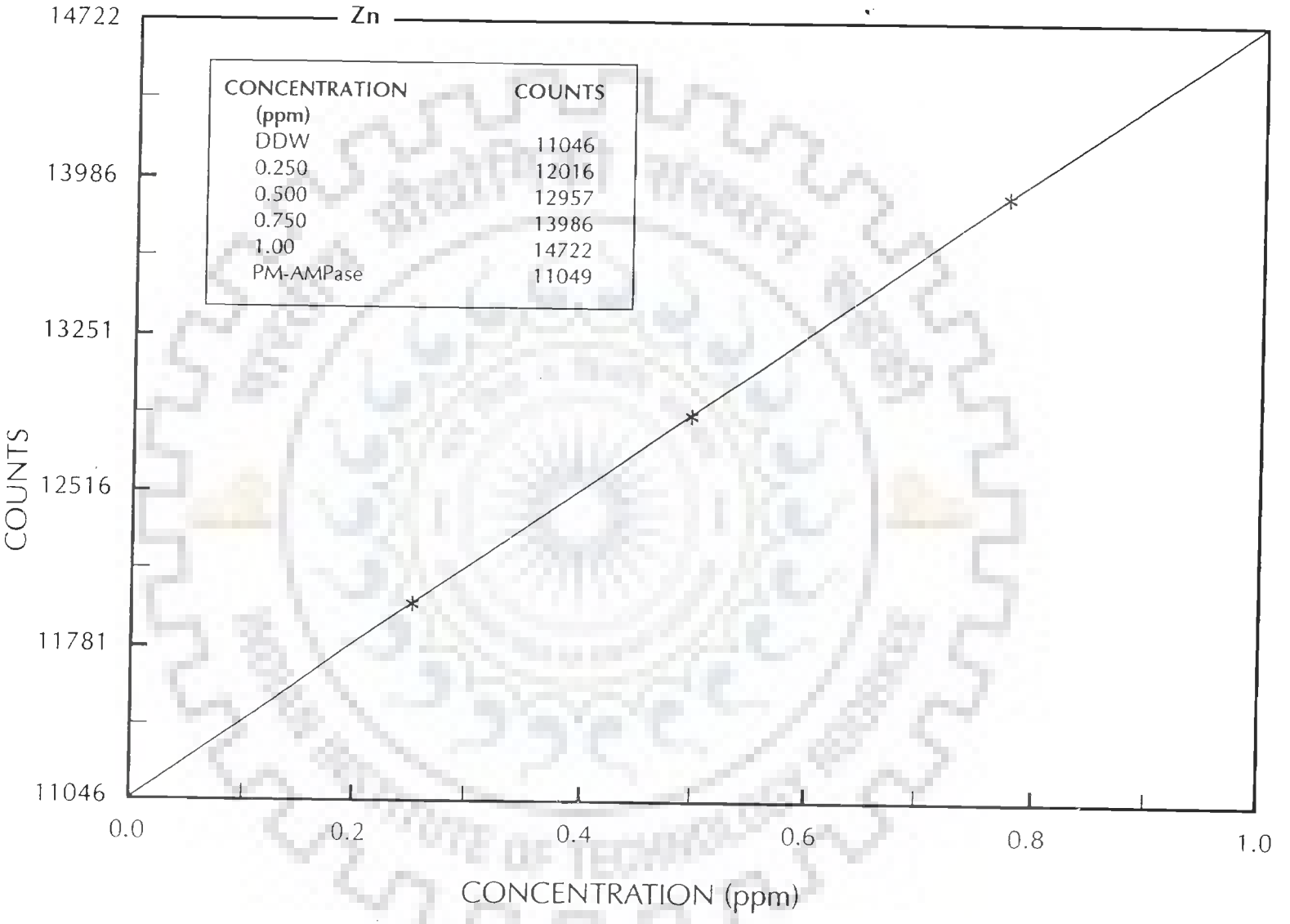


Fig. 4.15 ATOMIC ABSORPTION/EMISSON SPECTROSCOPY OF PURIFIED PM-AMPase
Purified PM-AMPase (500 µg) was suspended in 10.0 ml of double distilled water and its atomic absorption/emission was noted and compared with standard zinc solutions (0.250, 0.500, 0.750 and 1.00 ppm concentration) in the same matrix.

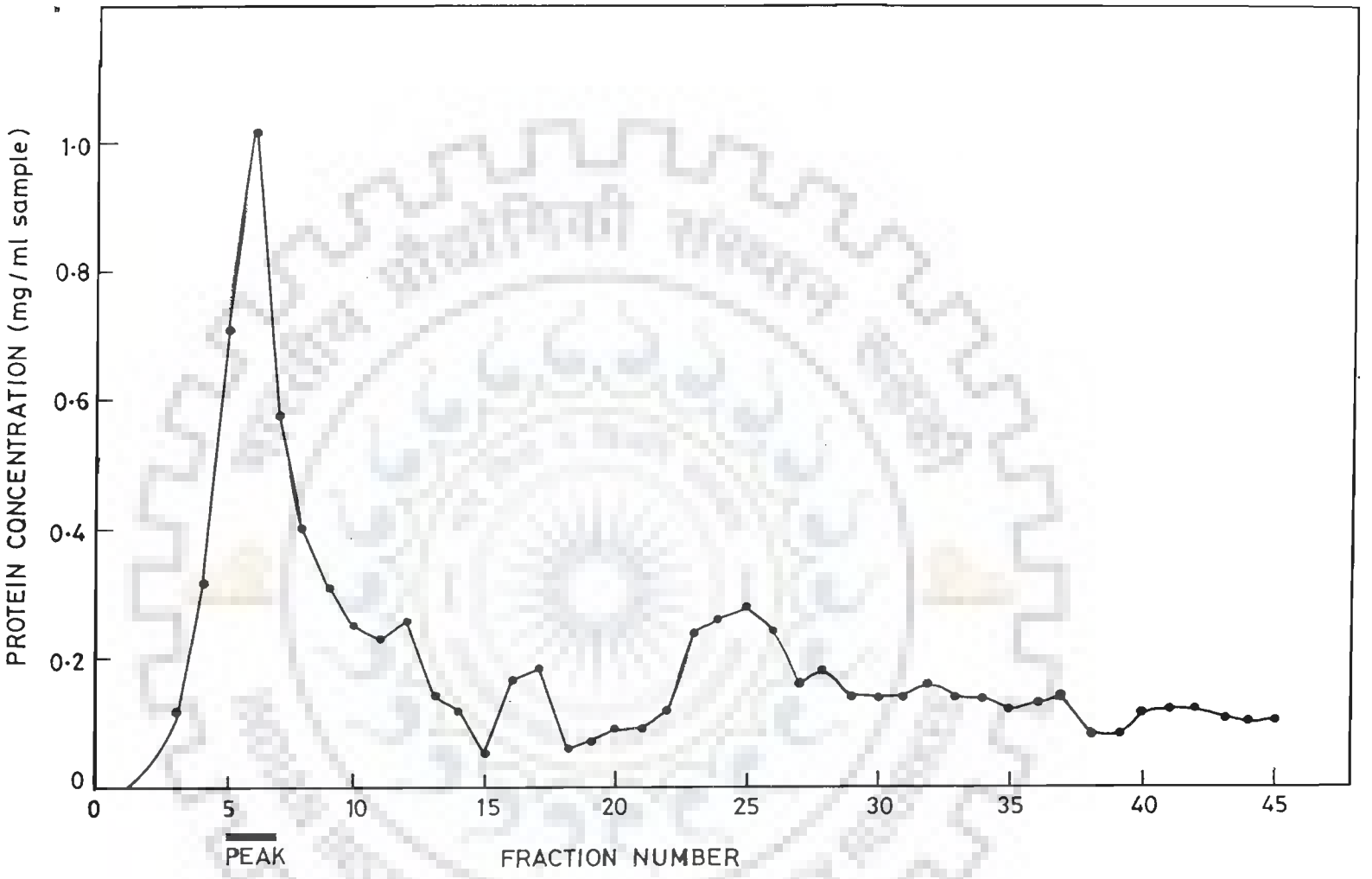


Fig.4.16 PURIFICATION OF IMMUNOGLOBULIN G (IgG)

The partial purification of antibodies was achieved by DEAE-cellulose column chromatography as described in Materials and Methods. IgG was obtained as unabsorbed and unsymmetrical peak (—) monitored by the protein concentration at 280 nm. 5ml of each fraction was collected in elution buffer 10 mM sodium-phosphate (pH 8.0) at flow rate of 0.25 ml/min. Fraction 5-7 represents the peak IgG fraction (—).

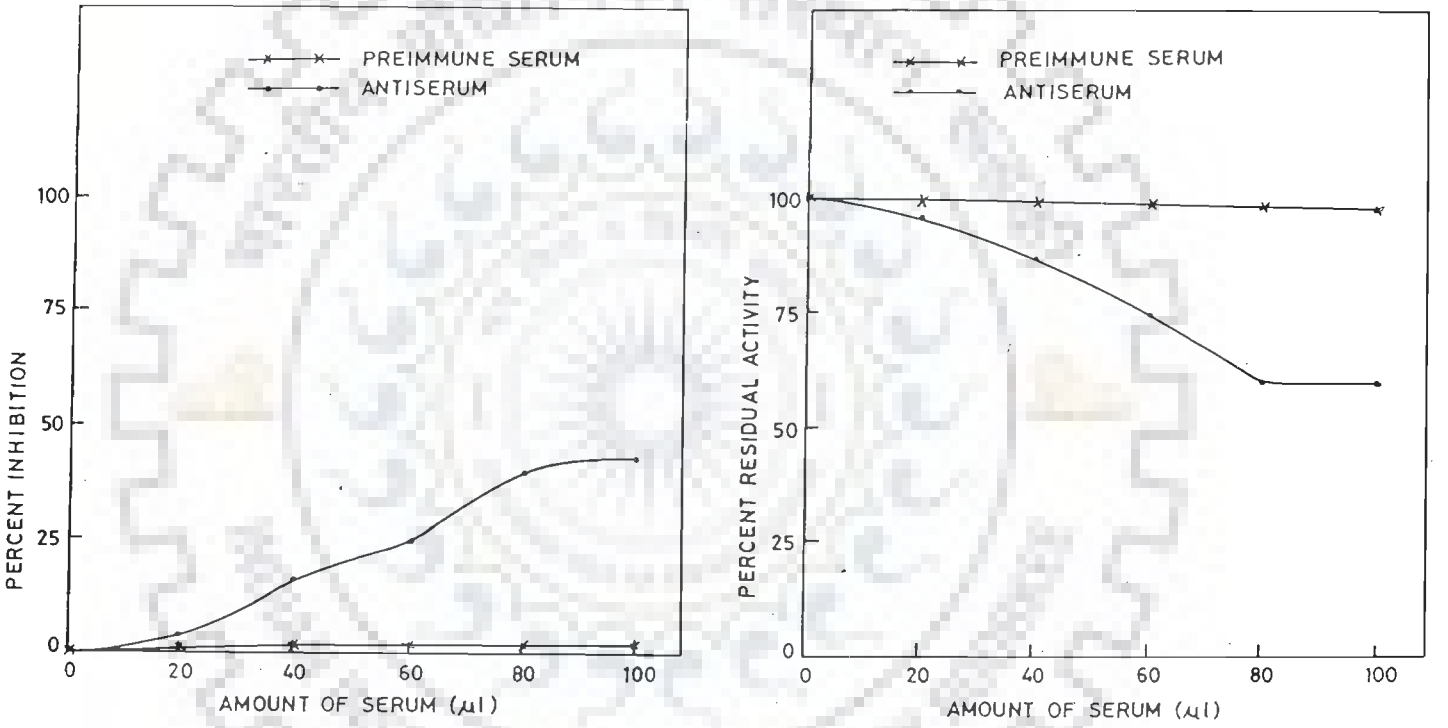


Fig.4.17 IMMUNOINHIBITION OF PLASMA MEMBRANE PM-AMPase BY ANTISERUM

Figure shows the extent of inhibition of plasma membrane PM-AMPase by antiserum. The preimmune serum (—x—x—) did not inhibit the enzyme activity. The percent residual activity of the enzyme decreased, i.e. the percent inhibition increased, with the increasing amount of antiserum (——).

enzyme means that in addition to the PM-AMPase, other non-specific acid phosphatases were also present in the detergent extract. On the basis of the inhibition data it can be assumed that nearly 45% of the total phosphatase activity in the detergent solubilized enzyme fraction can be accounted for by PM-AMPase. In fact, the immunological response of the PM-AMPase can provide a method for estimating the true proportion of the enzyme (PM-AMPase) in the crude extract.

No immunoprecipitation of the PM-AMPase activity by the preimmune serum from the detergent extract of the PM was noticed (Fig. 4.17), indicating further that the antibodies used were highly specific for the PM-AMPase and did not cross react with other phosphatases present in the PM. In other words, the PM-AMPase was immunologically different from other phosphatases of the peanut PM.

4.5.10 Peanut PM-AMPase Is Immunologically Different From PM-AMPase Of Other Plant Sources

The PM fractions from cotyledons of chickpeas *(Cicer arietinum)*, peas *(Pisum sativum)*, soybean *(Glycine max)* and wheat *(Triticum aestivum)* were prepared following the same protocol as was used for the preparation of peanut PM.

The membrane-bound AMPase was solubilized with detergent (CHAPS) and the enzyme activity in the crude detergent extract was determined. Although, the PM-AMPase activity was found in all PM fractions obtained from different plant sources, the specific activity of the PM-AMPase varied greatly from plant to plant. The maximum specific activity was exhibited by the peanut PM-AMPase followed by wheat (Table 4.6).

TABLE 4.6 PM-AMPase ACTIVITY FROM COTYLEDONS OF 7 DAYS OLD GERMINATING PLANT SEEDLINGS

Plant Source[b]	PM-AMPase Activity (μ mole Pi/min/mg-protein)[a]	
	Membrane - bound	Detergent solubilized
Peanut (*Arachis hypogaea*)	17.8	24.9
Pea (*Pisum sativum*)	15.9	3.5
Chickpeas (*Cicer arietinum*)	19.0	2.2
Soybean (*Glycine max*)	20.2	4.3
Wheat (*Triticum aestivum*)	54.15	17.6

a. Crude microsomal pellet (12,000-105,000xg pellet) was used for detergent solubilization of the AMPase activity.
b. Represents cotyledons of 7 days old germinating plant seedlings.

The immunological cross reactivity of various plant PM-AMPases was investigated by Western blotting using purified peanut PM-AMPase polyclonal mono-specific antibodies. Surprisingly, none of the plant PM-AMPases tested, except for the peanut PM-AMPase, cross reacted with the purified peanut PM-AMPase antibodies (Fig. 4.18). These results show that the peanut PM-AMPase was immunologically different from other plant PM-AMPases.

4.6 GOLGI APPARATUS AMPase (GA-AMPase)

4.6.1 Purification Of GA-AMPase

The subcellular distribution of AMPase in peanut cotyledon cells (Table 4.2) showed that at least one-third of the total microsomal enzyme was associated with GA and SER, which was essentially free from the contamination of PM fraction as indicated by the distribution of marker enzymes (Table 4.1). In order to compare the GA-AMPase with PM-AMPase, the former was prepared to homogeneity following the same purification protocol as was used for the purification of the PM-AMPase. The purification data are summarized in Table 4.7. The enzyme was released from the membrane by treatment with 1% (w/v) CHAPS. In terms of activity, nearly 50% of the membrane-bound AMPase was released by detergent extraction and the specific activity of the enzyme increased to 41.2 μ mole Pi/min/mg-protein from 17.6 μ mole Pi/min/mg-protein giving a purification of 2.3-fold. Thus, there seems to be a kind of selective solubilization of GA-AMPase by CHAPS since the protein solubilization was about 20-23% only. Under identical conditions the efficiency of the detergent in solubilizing GA-AMPase was significantly lower than PM-AMPase. In addition, the purification of GA-AMPase during solubilization step was only 2.3-fold compared to about 5-fold for the PM-AMPase. On Sephadex G-150 the GA-AMPase was eluted in a single symmetrical peak (fractions 56 to 59) at an elution volume of 116 ml (Fig. 4.19). The overall purification and yield of the GA-AMPase relative to the GA fraction was approximately

Fig.4.18 CROSS REACTIVITY WITH OTHER PLANT SOURCES PM-AMPase

The photograph shows the protein pattern obtained after the Western blotting of the wheat, chickpea, pea, soybean and peanut membrane fractions using the antibodies against the peanut PM-AMPase. Various lanes represent :

A : Chickpea AMPase
B : Pea AMPase
C : Soybean AMPase
D : Wheat AMPase
E : Peanut AMPase
F : Positive protein (Rabbit Immunoglobulin)

TABLE 4.7 PURIFICATION OF GA-AMPase FROM DIFFERENT FRACTIONS OF COTYLEDONS OF 7-DAYS OLD GERMINATING PEANUT SEEDLINGS

Fraction	Total Protein[a] (mg)	Total AMPase Activity[a] (μ mole Pi/min)	Specific Activity of AMPase (μ mole Pi/min/ mg-protein)	Purification (fold)	Yield (%)
Enriched GA (37/25% sucrose interface)	62.47	1100.1	17.6	-	100.00
CHAPS[b] Extract (105,000 xg supernatant)	14.30	588.3	41.2	2.34	53.5
Gel Filtration (Sephadex G-150)	1.20	218.4	182.0	10.33	19.8
DEAE - Cellulose	0.31	19.5	62.8	3.57	1.8

a. data represent 400g fresh weight tissue
b. (3-[(3-Cholamidopropyl) - dimethylammonio] -1- propane-sulfonate)

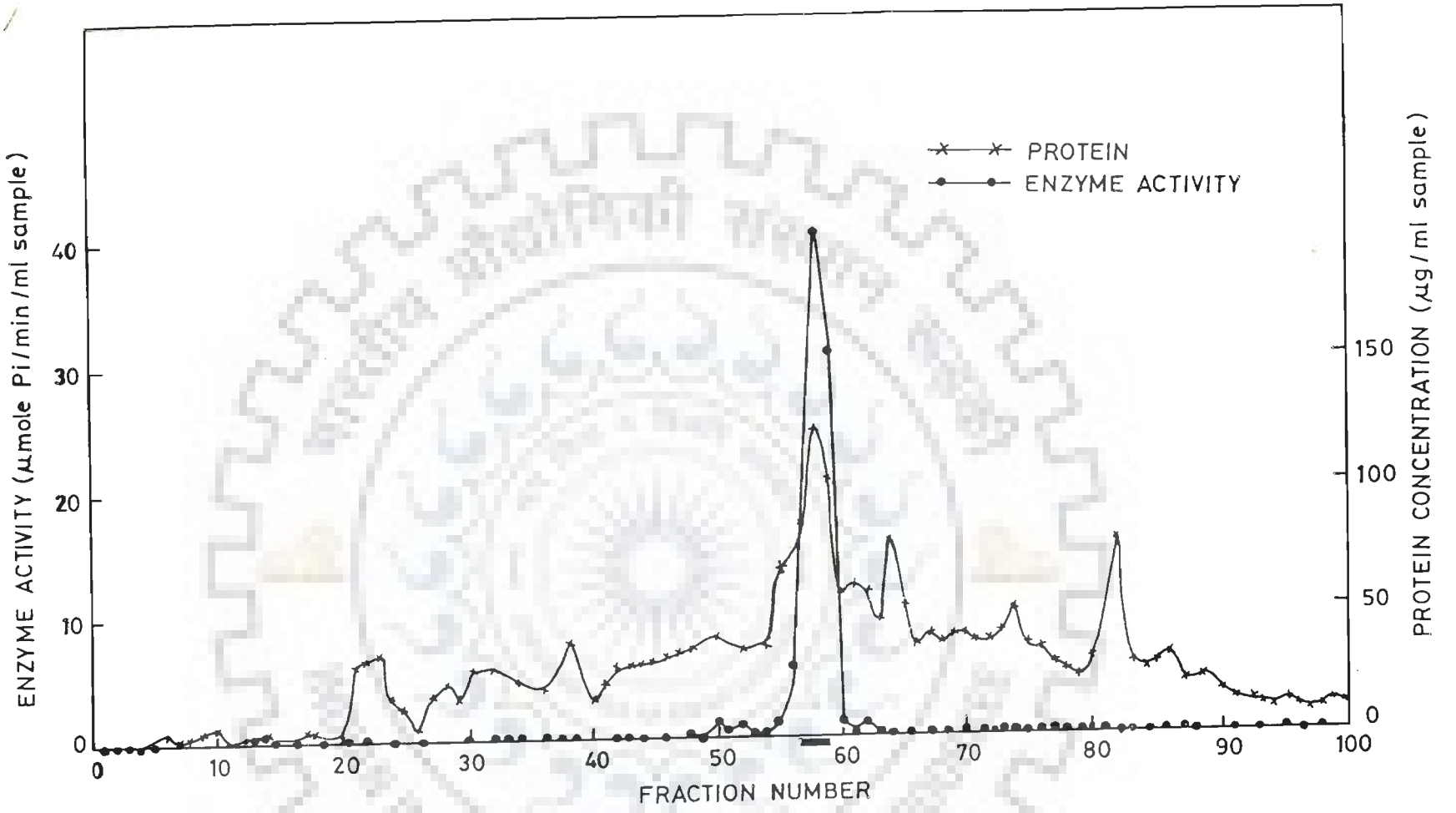


Fig.4.19 PURIFICATION OF GA-AMPase ON SEPHADEX G-150 COLUMN

This is an elution profile of the chromatography of CHAPS solubilized enzyme dialyzed for 6 h against 10mM Tris-HCl (pH 7.2) with 0.04% β-mercaptoethanol and 0.01% CHAPS. The enzyme was loaded on pre-cooled and pre-equilibrated Sephadex G-150 column (1x100 cm). Fractions of 2ml each were collected at flow rate of 1ml/20 min. The enzyme eluted in one major peak (57 to 59) (▬). The enzyme activity and protein concentration were determined as described in Material and Methods.

10-fold and 20%, respectively. In addition, like PM-AMPase it was quite active and stable at this stage. As judged from the elution volumes at which GA-AMPase and PM-AMPase were eluted, the former appeared to be a higher molecular weight enzyme than the latter. The final step of purification was ion-exchange chromatography on DEAE-cellulose column. The elution profile is shown in Fig. 4.20. The enzyme eluted as a single peak, comprising fractions 15 to 17, at NaCl gradient of 100mM. The PM-AMPase was eluted at 130mM. Thus, on the basis of chromatographic behaviour, the GA-AMPase appeared to be different from the PM-AMPase. However, like PM-AMPase, the GA-AMPase was also rendered unstable after the DEAE - cellulose column, as indicated by sharp decline in the total activity as well as specific activity. However, in the absence of inactivation of the enzyme during DEAE-cellulose chromatography, the actual purification fold would be much greater than the indicated value.

4.6.2 Homogeneity Of GA-AMPase

The homogeneity of GA-AMPase obtained from the DEAE-cellulose column (final step of purification scheme) was tested by SDS-PAGE under fully-denaturing conditions. For comparison detergent solubilized and purified enzymes from both GA and PM were run simultaneously on SDS-PAGE. It can be seen that both GA-AMPase and PM-AMPase moved on the gel as single sharp protein bands corresponding to apparent molecular masses of 52,400 and 26,300 daltons, respectively (Fig. 4.21). These results together with the chromatographic behaviour clearly demonstrate that the GA-AMPase and PM-AMPase are two different enzymes. Fig. 4.22 shows the log molecular weight of standard proteins plotted against the R_f values to deduce the molecular weight of GA-AMPase and PM-AMPase after SDS-PAGE. It may be pointed out that since the SDS-PAGE was performed under fully-denatured conditions, the two enzymes represent single polypeptide subunits of

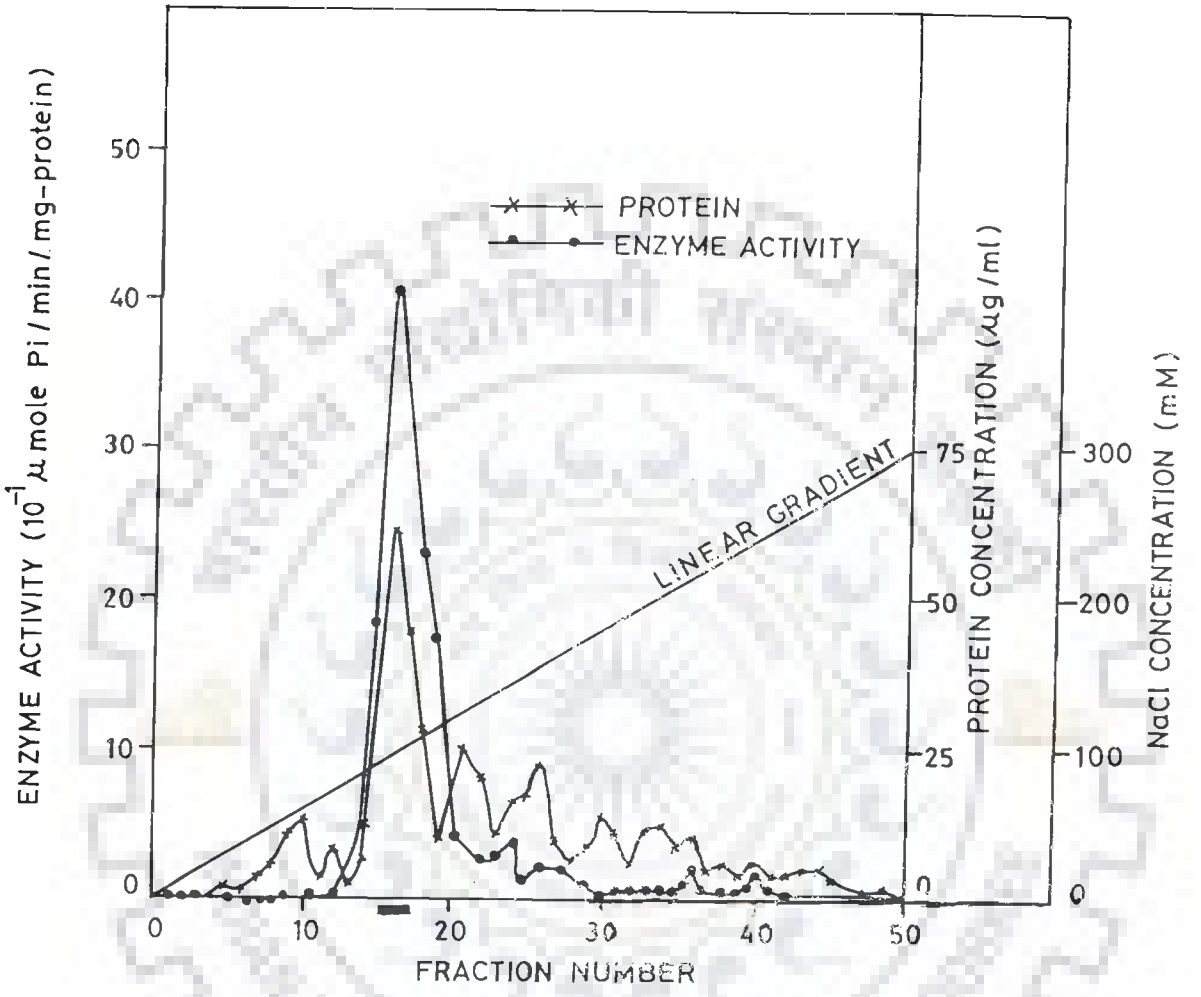


Fig.4.20 ELUTION PROFILE OF GA-AMPase FROM DEAE-CELLULOSE COLUMN

The enzyme from Golgi apparatus was purified using the same protocol as for plasma membrane. The pooled peak enzyme after gel filtration column was loaded on DEAE-cellulose column (1x1.5 cm). The absorbed proteins were eluted by a linear gradient of NaCl (0-300 mM) in 10mM Tris-HCl, pH 7.2, with 0.04% β-mercaptoethanol, 0.01% CHAPS and 20% glycerol. Fractions of 2 ml each were collected at flow rate of 0.2 ml/min and analyzed for protein content and enzyme activity. Elution profile shows enzyme activity obtained at 100 mM NaCl concentration. The major peak (—) constituted fractions 15-17.

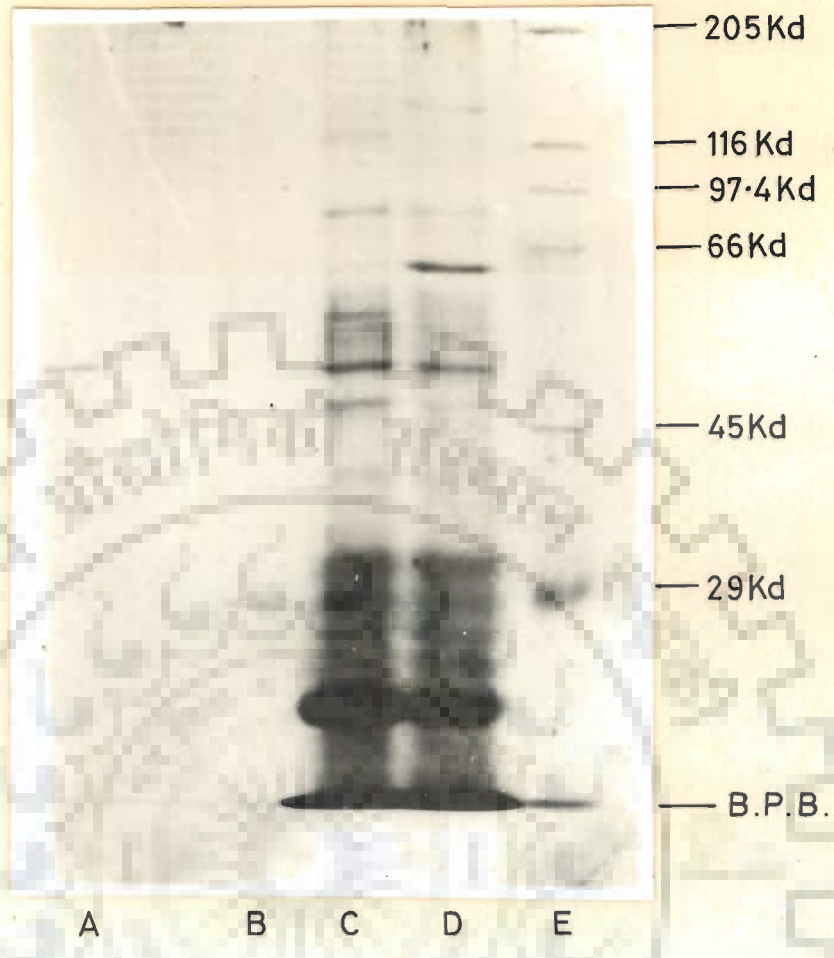


Fig.4.21 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED GA-AMPase AND PM-AMPase

SDS-Polyacrylamide gel electrophoresis was carried out as described in Material and Methods with purified AMPase from PM and GA fractions along with PM and GA solubilized membrane fractions and standard molecular weight markers. Photograph shows

A : Purified AMPase from GA
B : Purified AMPase from PM
C : GA solubilized enzyme fraction
D : PM solubilized enzyme fraction
E : standard molecular weight markers as:
29 Kd, Carbonic anhydrase; 45 Kd, Egg albumin;
66 Kd, Bovine Albumin, ; 97.4 Kd, Phosphorylase B;
116 Kd; β-Galactosidase; 205 Kd, Myosin.

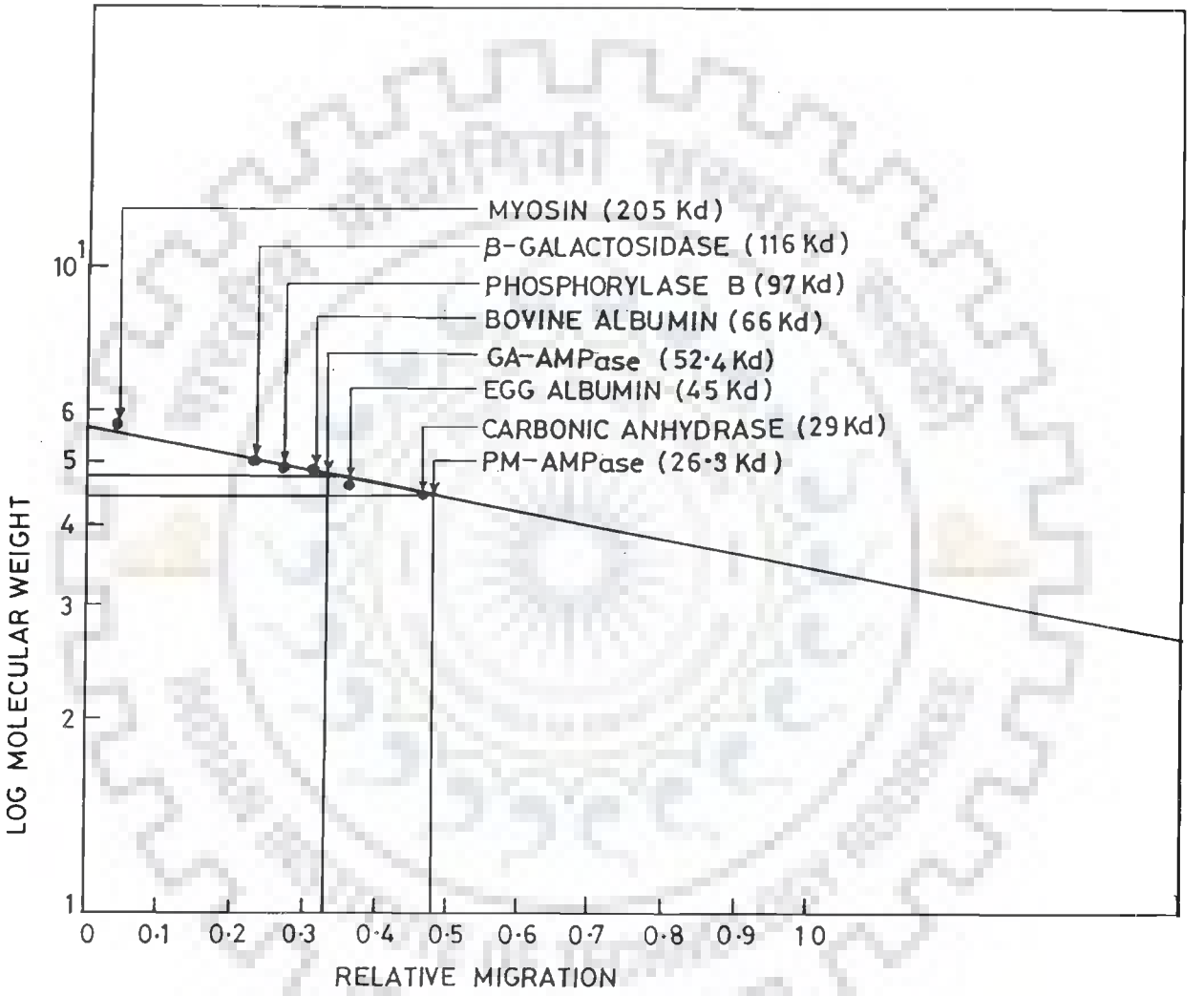


Fig.4.22 ESTIMATION OF MOLECULAR WEIGHT OF PURIFIED GA-AMPase

SDS -Polyacrylamide gel electrophoresis was carried out on 10% gel in fully-dissociating and denaturing conditions as described in Material and Methods . Mobilities were determined relative to bromophenol blue, as tracking dye.

molecular masses 52,400 and 26,300 daltons (Fig. 4.22). As such GA-AMPase may not be a dimeric form of the PM-AMPase.

The native gel profile under non-denaturing conditions showed that GA-AMPase activity concentrated in a single peak near the bovine serum albumin (66 Kd) band (Fig. 4.23). Since under the non-denaturing conditions dissociation of the enzyme into subunits is unlikely, the single peak of the GA-AMPase on the native gel lends further support to the SDS-PAGE data that the GA-AMPase is a higher molecular mass (52.4 Kd) single polypeptide chain, as compared to PM-AMPase which is a lower molecular mass (26.3 Kd) single polypeptide chain. Thus, the data clearly suggests that the GA-AMPase is a different enzyme and as such may not be related to the PM-AMPase. More detailed study, however, will be required to establish the structural differences between the GA-AMPase and the PM-AMPase.

4.6.3 Response Of GA-AMPase Towards PM-AMPase Antibodies

In order to investigate the immunological relationship, the GA-AMPase was tested against the PM-AMPase antibodies. Interestingly, double immunodiffusion and Western blots of GA-AMPase (Fig. 4.24 and Fig. 4.25) showed no cross reactivity with the PM-AMPase polyclonal monospecific antibodies, indicating once again that GA-AMPase did not share the common determinants and is an immunologically different enzyme. The results of immunoprecipitation of GA-AMPase by PM-AMPase antibodies (Fig. 4.26) also show that the enzymes from the PM and GA are immunologically different. For instance, there was no significant inhibition of GA-AMPase activity by the PM-AMPase antibodies. In contrast, the activity of PM-AMPase was inhibited even in crude form by the antiserum. As expected, the preimmune serum showed no inhibition of GA-AMPase activity.

4.7 PROPERTIES OF GA-AMPase

4.7.1 Optimum pH

The activity profile of the purified GA-AMPase as a function of pH is shown in Fig. 4.27. The optimum pH of the enzyme was found to be in the range of 5.0 to 5.5. The activity of the enzyme outside the optimum pH range decreased rapidly.

4.7.2 Kinetic Parameters

The effect of substrate concentration on the activity of the enzyme was determined with the help of double reciprocal plot (Fig. 4.28). A linear relationship between the reciprocal of velocity and reciprocal of substrate concentration was found. The K_m and V_{max} for the hydrolysis of 5'-AMP by GA-AMPase were found to be approximately 1.02mM and 6.85 μ mole Pi/min/mg-protein, respectively.

4.7.3 Substrate Specificity

The results of substrate specificity of the purified GA-AMPase are given in Table 4.8. Under the standard AMPase assay conditions, the purified GA-AMPase exhibited highest substrate specificity towards 5'-AMP followed by 5'-GMP, p-nitrophenyl phosphate (p-NPP) and 5'-UMP. For example, the efficiency of the hydrolysis of 5'-GMP, p-NPP and 5'-UMP relative to 5'-AMP hydrolysis by the enzyme were approximately 45%, 38% and 16%, respectively. Thus, as compared to the substrate specificity of PM-AMPase (Table 4.4), the GA-AMPase is less specific and also catalyzes the hydrolysis of 5'-GMP, p-NPP and 5'-UMP to a significant extent. Since the enzyme is homogeneous as shown by SDS-PAGE, the hydrolysis of 5'-GMP, p-NPP and 5'-UMP may be due to non-specific nature of the phosphatase activity of GA-AMPase and certainly does not seem to be due to contamination of GMPase, UMPase and acid phosphatase.

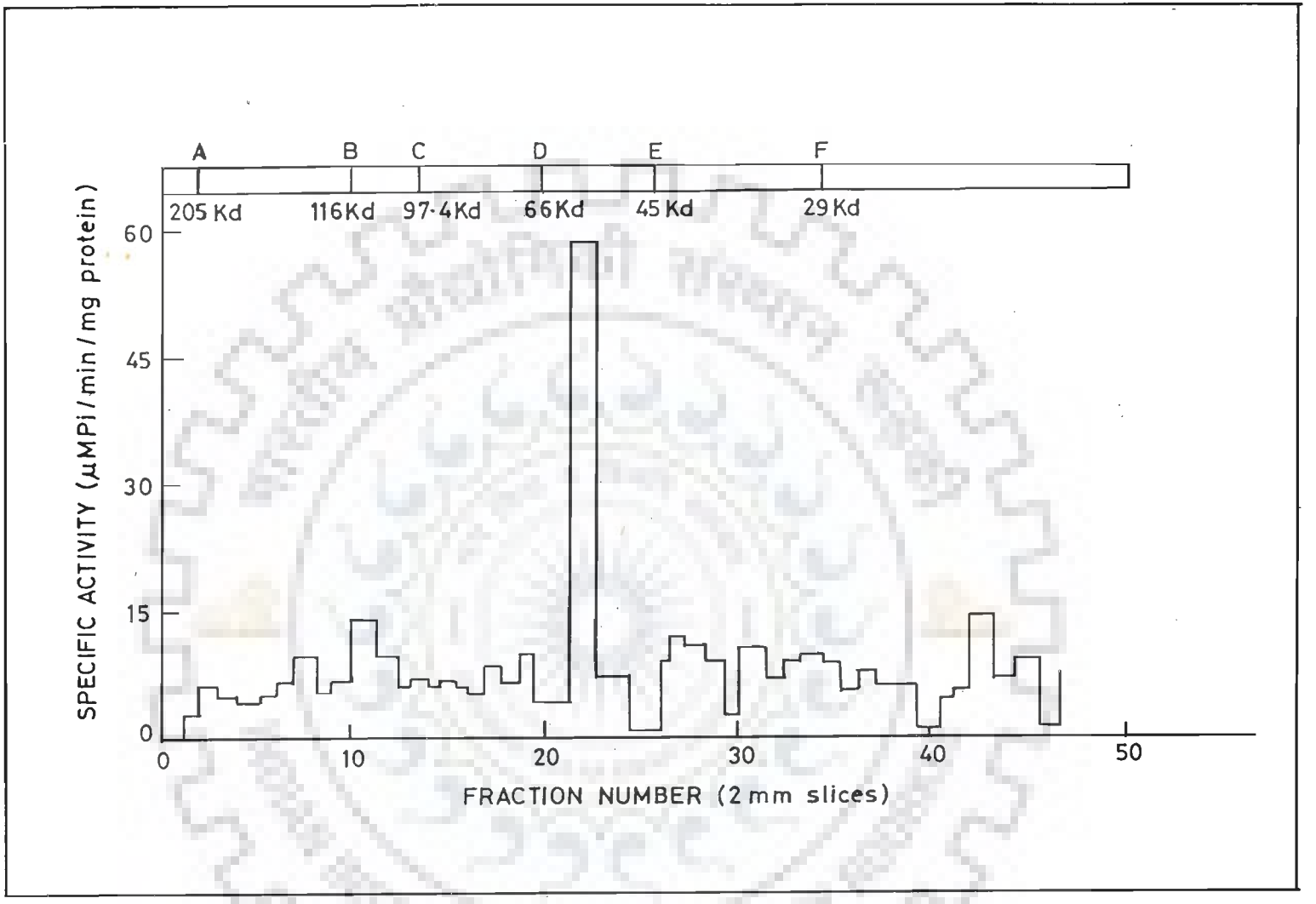


Fig.4.23 NATIVE GEL PROFILE FOR PURIFIED GA-AMPase

Polyacrylamide gel electrophoresis was carried out without SDS at 4^{0} C under non-denaturing conditions. 2 mm slices were cut, homogenized in 50 mM sodium-acetate buffer (pH 5.0) and centrifuged. The supernatant was assayed for enzyme activity using standard protocol as described in Material and Methods. A,B,C,D, E and F are standard proteins used. A: Myosin; B: β-Galactosidase; C: Phosphorylase B; D: Bovine albumin; E: Egg albumin; F:Carbonic anhydrase.

Fig.4.24 DOUBLE IMMUNODIFFUSION WITH GA-AMPase
Conditions used for immunodiffusion were those as described under Material and Methods. As seen in the photograph the different wells contained.

Well 1 : PM-AMPase
Well 2 : Immune Serum
Well 3 : GA-AMPase

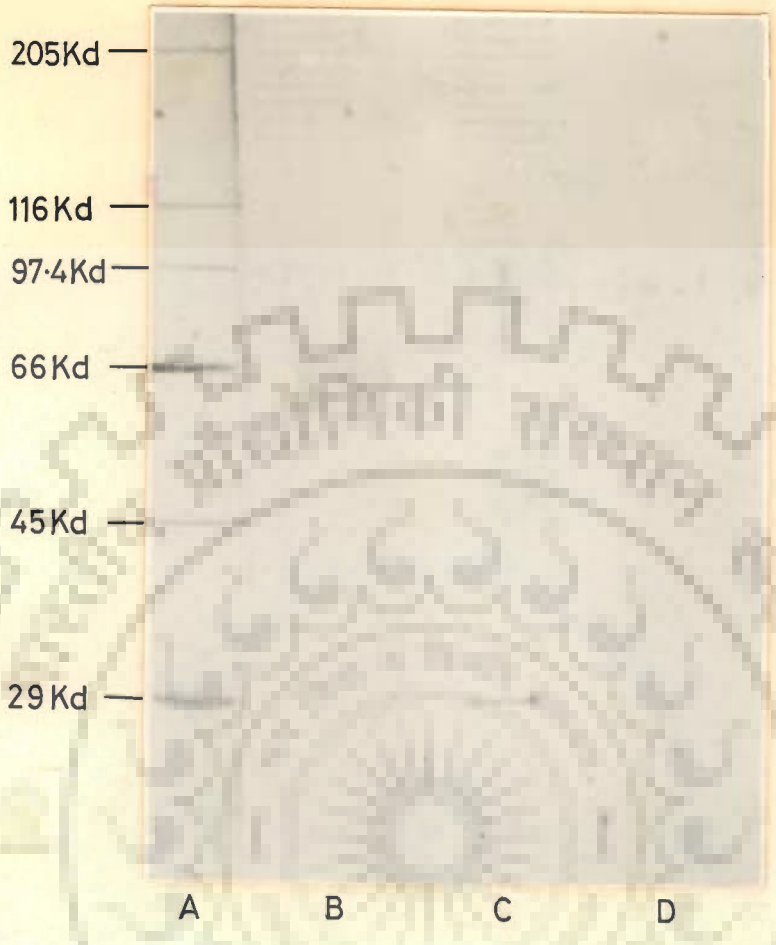


Fig.4.25 WESTERN BLOTTING EXPERIMENT WITH GA-AMPase

SDS-PAGE was carried out as described in Material and Methods with the AMPase purified from GA. Proteins were transferred to nitrocellulose sheet after electrophoresis using semi-dry transfer apparatus as described in Material and Methods and then the nitrocellulose sheet was cut from the side of molecular weight marker to check the transfer. Rest of the proteins were subjected to Western blotting using antibodies raised against purified PM-AMPase. Amersham kit was used. Photograph shows

A : standard molecular weight markers as:
29 Kd, Carbonic anhydrase; 45 Kd, Egg albumin;
66 Kd, Bovine Albumin, ; 97.4 Kd, Phosphorylase B;
116 Kd; β-Galactosidase; 205 Kd, Myosin.

B : Positive protein

C : PM-AMPase

D : GA-AMPase

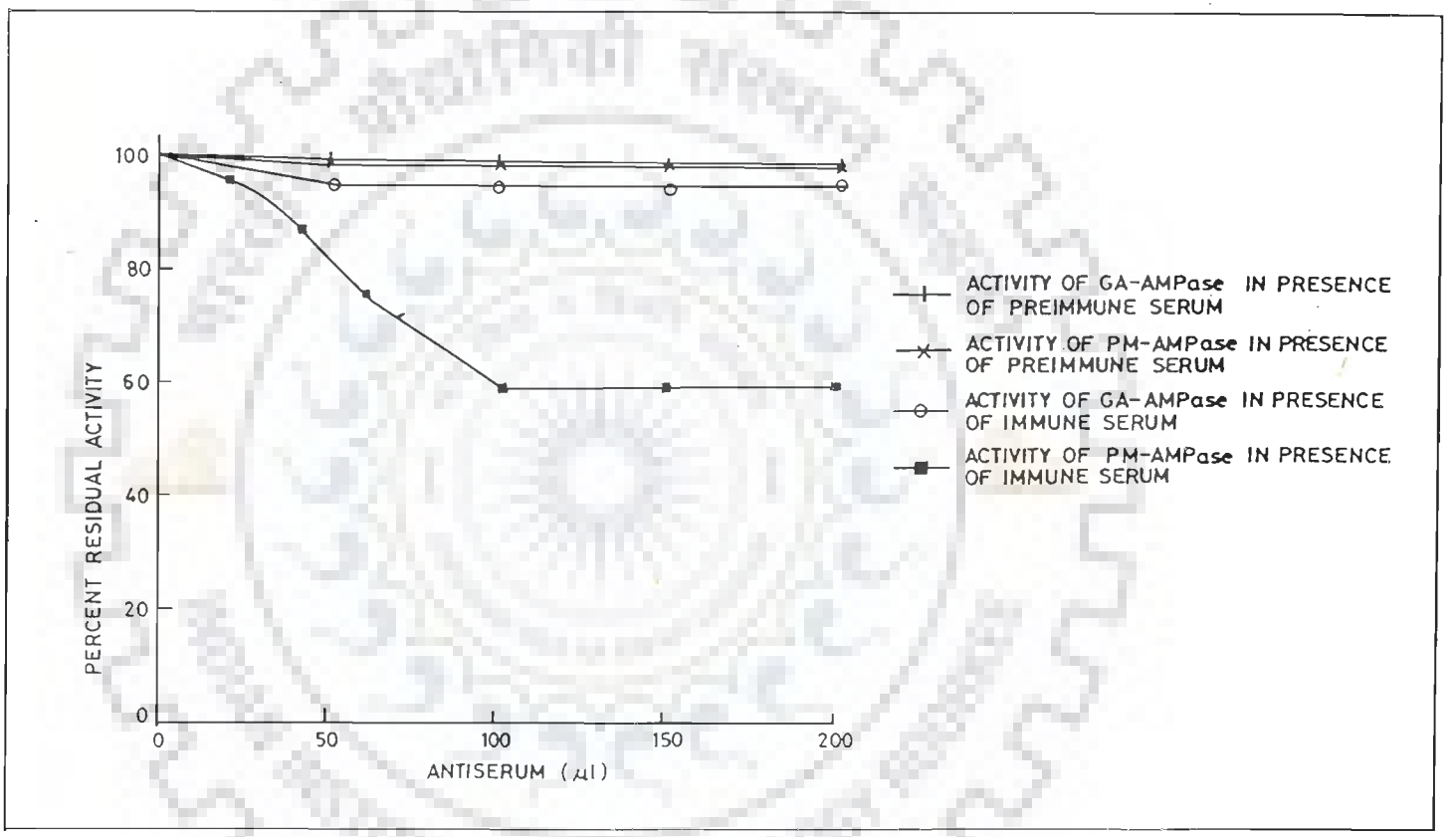


Fig.4.26 IMMUNOINHIBITION OF GA-AMPase BY PM-AMPase ANTISERUM

Figure shows the extent of inhibition of the GA-AMPase and PM-AMPase in the solubilized enzyme fraction by the antibodies raised against the purified PM-AMPase. The GA-AMPase in the solubilized enzyme fraction did not show any inhibition in enzyme activity with immune and preimmune serum while PM-AMPase enzyme activity was inhibited by immune serum.

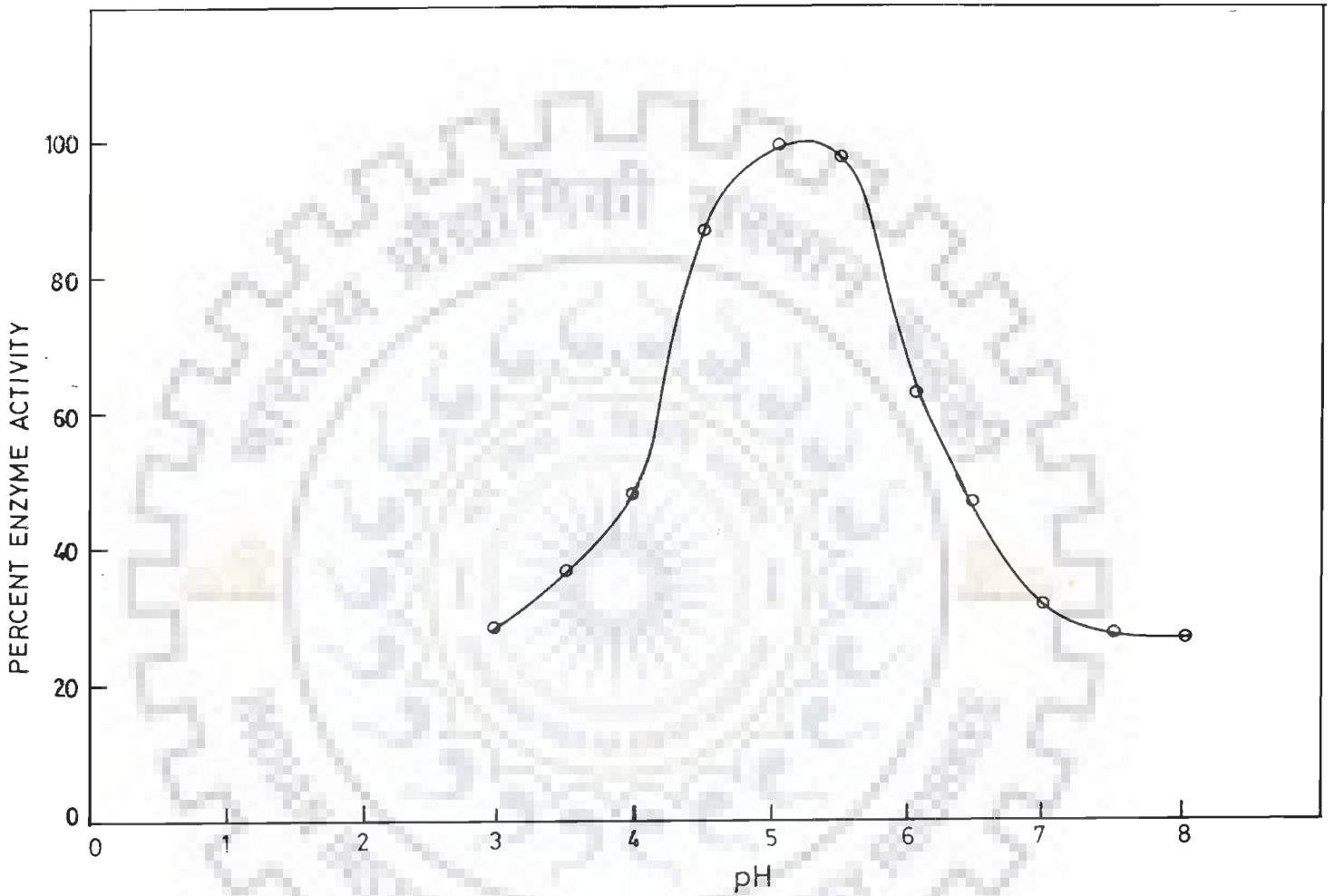


Fig.4.27 pH OPTIMA OF PURIFIED GA-AMPase

The enzyme activity was measured in the standard assay conditions except that the buffer and pH were varied. Buffers used were 50 mM sodium-acetate (pH 3.0 to 5.5) and 50 mM Tris-HCl (pH 6.0 to 8.0). Maximum enzyme activity was taken as 100%.

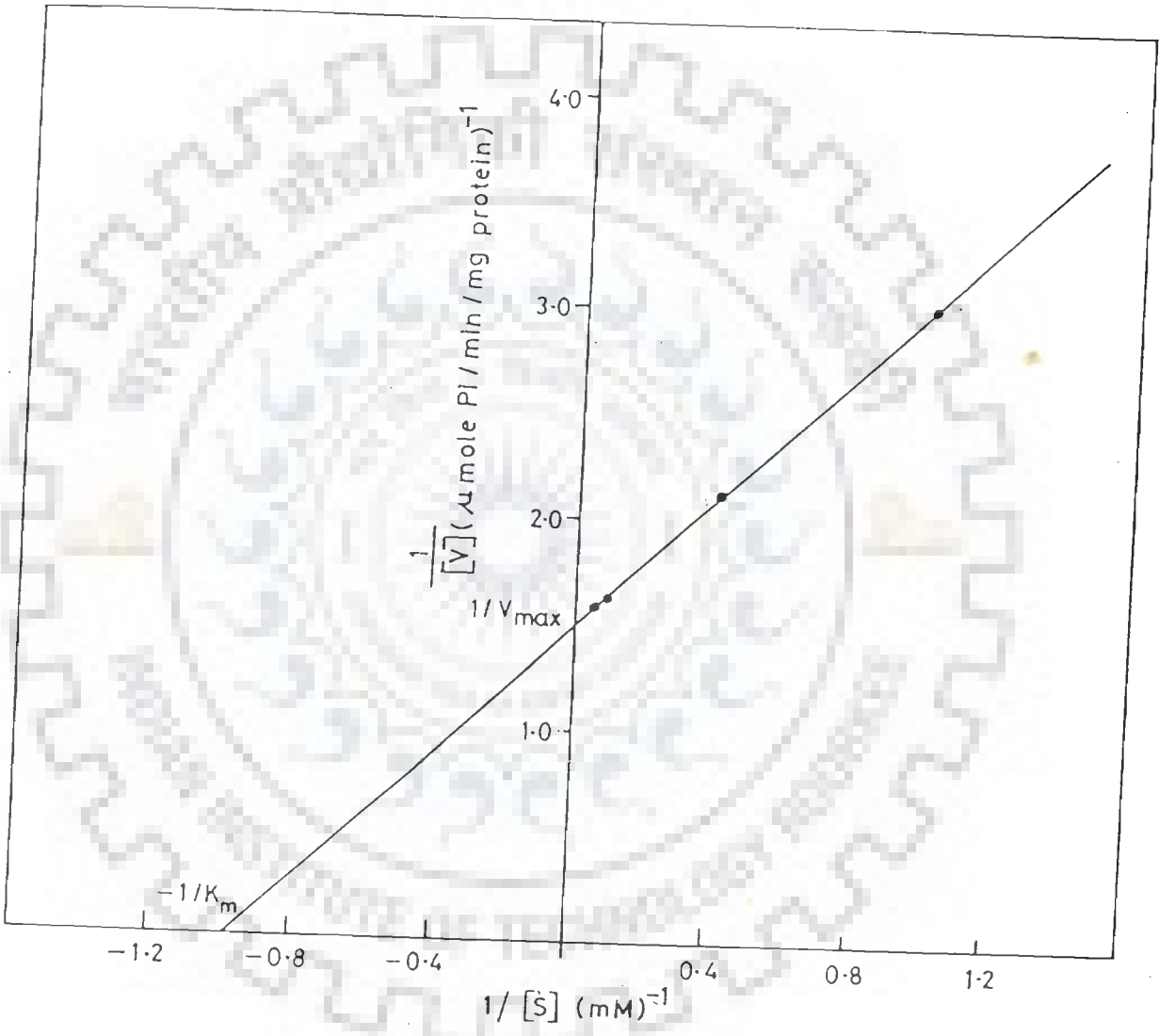


Fig.4.28 LINEWEAVER-BURK PLOT OF GA-AMPase

This is the Lineweaver-Burk plot of purified GA-AMPase using 5'- AMP as substrate in 50 mM sodium-acetate buffer, pH 5.0. Enzyme was assayed at different concentrations of substrate under standard assay conditions. K_m and V_{max} values were computed from the intercepts on X-axis and Y-axis, respectively.

TABLE 4.8 SUBSTRATE SPECIFICTY OF PEANUT GA-AMPase

Substrate	Purified GA-AMPase Activity	
	(μ mole Pi/min/ mg-protein)	(%)
Adenosine-5′-monophosphate	62.87	100.00
Adenosine-5′-diphosphate	3.16	5.02
Adenosine-5′-triphosphate	1.83	2.91
Guanosine-5′-monophosphate	28.91	45.98
Guanosine-5′-diphosphate	3.01	4.78
Uridine -5′-monophosphate	10.16	16.16
Uridine -5′-diphosphate	2.96	4.71
Glucose -1-phosphate	0.93	1.47
Glucose -6-phosphate	1.17	1.86
p-Nitrophenylphosphate	24.01	38.19

4.7.4 Carbohydrate Content

The purified enzyme was dialyzed exhaustively against distilled water and lyophilized. The protein content was determined after dissolving in minimum volume of distilled water and the carbohydrate content was determined by phenol-H_2SO_4 method (Dubois et al., 1956). The carbohydrate content was computed by referring to standard plot (Fig. 4.29). The dialyzed enzyme was found to contain about 39.1% carbohydrate, which was apparently covalently bound to the protein of the enzyme.

4.7.5 Effect Of Phospholipids

The enzyme activity of the purified GA-AMPase was significantly enhanced by phospholipids (Table 4.9). The maximum activation of the enzyme activity was obtained with phosphatidylcholine followed by phosphatidylserine and phosphatidylglycerol. Since freshly purified enzyme was active even in the absence of phosphatidylcholine, the enzyme may not require phosphatidylcholine for its activity. However, as pointed out earlier in the case of PM-AMPase, may be the presence of phospholipids, particularly phosphatidylcholine, helps in stabilizing the enzyme and protects it from denaturation once the enzyme has been solubilized from the membrane, the natural environment of the enzyme.

4.8 COMPARISON OF PLASMA MEMBRANE AND GOLGI APPARATUS-AMPases

A comparison of the properties of the purified enzymes is shown in Table 4.10. The two enzymes resemble in subunit structure, optimum pH range, activation by phosphatidylcholine and carbohydrate content. There are, however, some striking differences in the properties of the two enzymes, for example, the PM-AMPase is a low molecular mass (26.3Kd) while the GA-AMPase is a high molecular mass species (52.4Kd). The K_m values with 5'-AMP as substrate are also significantly different, for example, PM-AMPase has K_m of 0.58mM compared to 1mM of GA-AMPase. In other

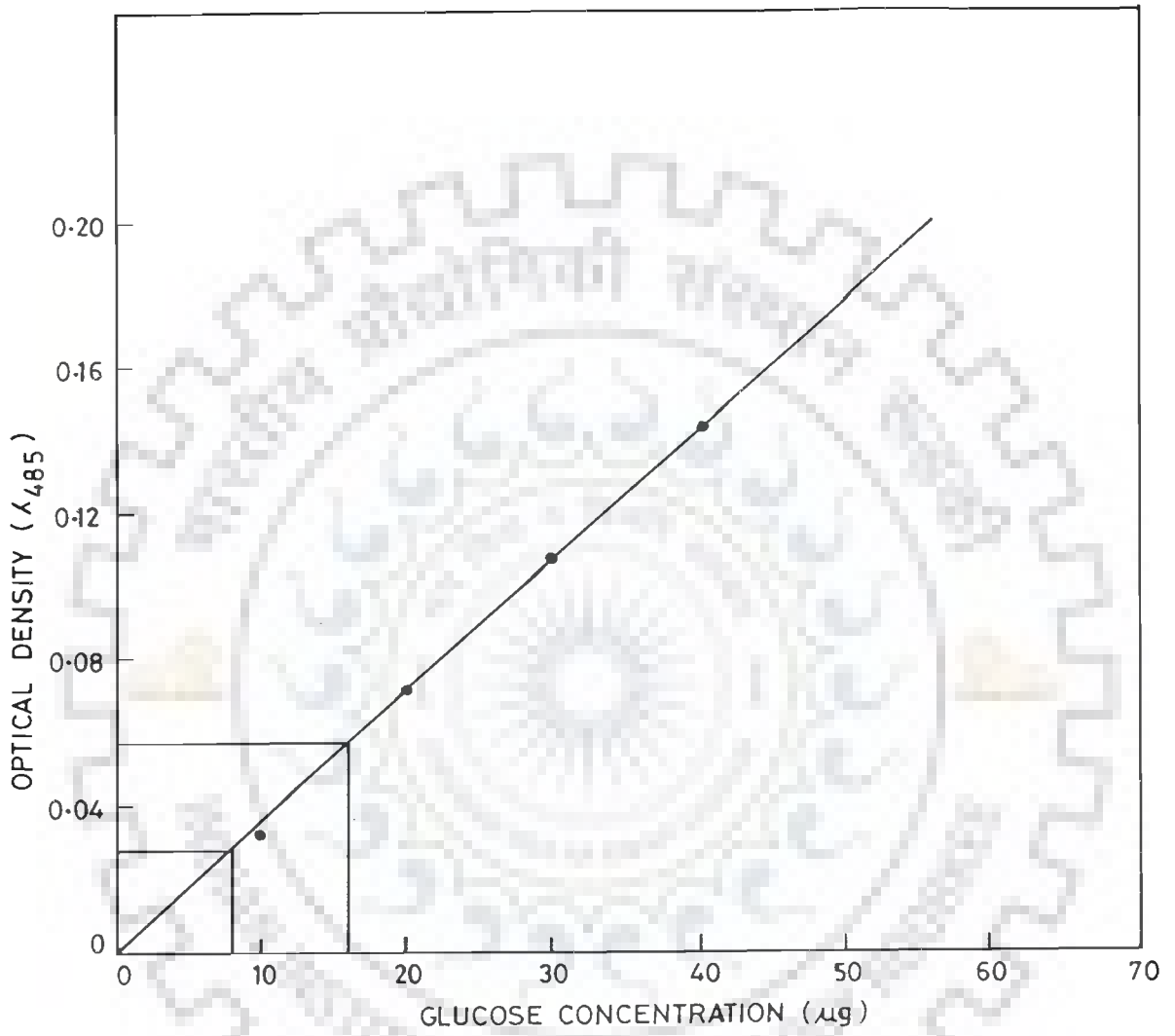


Fig.4.29 ESTIMATION OF CARBOHYDRATE CONTENT OF PURIFIED GA-AMPase.
The protein was dialyzed overnight against double distilled water, lyophilized and suspended in minimum amount of distilled water. The protein content and the carbohydrate content were determined from the standard curve. The carbohydrate content of the purified GA-AMPase was found to be 39.1%.

TABLE 4.9 EFFECT OF DIFFERENT PHOSPHOLIPIDS ON THE RESTORATION OF THE ACTIVITY OF PARTIALLY ACTIVE GA-AMPase

Phospholipid Added	GA-AMPase[a] Specific Activity (μ mole Pi/min/ mg-protein)	Percent of Control
None	11.42	100
Phosphatidylcholine	15.13	132.48
Phosphatidylserine	12.43	118.85
Phosphatidylglycerol	13.04	111.42

a. The GA-AMPase obtained from DEAE-cellulose column (specific activity 62.87μ mole Pi/min/mg-protein) was stored in glycerol buffer at -20°C. During storage about 82% of the enzyme activity was lost. The partially active enzyme (specific activity 11.42 μ mole Pi/min/mg-protein) was pre-incubated with 2μg/ml phospholipid in ice for 15 min before assaying the activity by standard procedure. Control assays were performed concurrently without adding phospholipid in incubation mixture.

TABLE 4.10 A COMPARISON OF THE PROPERTIES OF PURIFIED AMPases FROM PM AND GA-FRACTIONS OF PEANUT COTYLEDONS

Properties	PM-AMPase	GA-AMPase
molecular mass	26.3Kd	52.4Kd
Subunits present	Single polypeptide	Single polypeptide
pH optima	5.0-5.5	5.0-5.5
K_m	0.58mM	1.02mM
V_{max}	5.9 μ mole Pi/min/mg	6.85 μ mole Pi/min/mg
Substrate specificity	Highly specific for 5'-AMP only	Highly specific for 5'-AMP and hydrolyzes 5'-GMP, 5'-UMP and pNPP as well
Elution from DEAE-cellulose column	130mM	100mM
Effect of phospho-lipids on enzyme activity		
Phosphatiylcholine	45% increase	32% increase
Phosphatidylserine	11% increase	18% increase
Phosphatidylglycerol	33% increase	11% increase
Carbohydrate content	37%	39%
Immunological response towards PM-AMPase polyclonal antibodies	yes	No

words, the affinity towards 5'-AMP of PM-AMPase is almost two times more than that of GA-AMPase. PM-AMPase is more specific for 5'-AMP and has no or little hydrolytic activity towards other nucleotides and sugar phosphates. In contrast, although GA-AMPase shows specificity for 5'-AMP, it also catalyzes the hydrolysis of 5'-GMP, 5'-UMP and p-NPP. In addition to these, the most striking difference is the response of the two enzymes towards antibodies raised against purified PM-AMPase. As expected, the PM-AMPase polyclonal monospecific antibodies show precipitation reaction on double immunodiffusion plates and also react with PM-AMPase on Western blots. Also the immunoprecipitation reaction of the PM-AMPase antibodies with the enzyme is indicated by the decrease in the enzyme activity in the supernatant that remained after removal of antibody-enzyme complex. Unexpectedly, however, the polyclonal antibodies prepared against the PM-AMPase showed no precipitation reaction whatsoever, against the purified GA-AMPase, indicating that the two enzymes are immunologically different and do not share common epitopes. This is an important observation and this, not only shows that the two enzymes may be the product of different genes but also suggests that the intracellular translocation of the two enzymes follow different routes. In other words, PM-AMPase migrates to the PM by default mechanism without being retained in the GA whereas the GA-AMPase is retained in GA and does not migrate to the PM. What kind of signal retains the GA-AMPase in the GA is not known at the moment.

Chapter 5

5.0 DISCUSSION

For several years our laboratory has been concerned with the characterization of higher plant membrane glycoprotein enzymes (Sharma et al.,1986; Basba et al., 1987; Mittal et al.,1988; Kamat et al., 1992). Sharma et al.(1986) and Mittal et al. (1988) had shown the presence of multiple forms of 5'-nucleotidase (AMPase) in both PM and GA membrane fractions from peanut cotyledons. They had also purified apparently dominant species of the enzyme from these fractions and showed that these were glycoproteins. Their work showed that the PM-AMPase and GA-AMPase exhibited fair degree of resemblance in their properties which led to the conclusion that the GA-AMPase may be the precursor of PM-AMPase and that the two enzymes were structurally related. However, no immunological studies were performed. Further, other forms of the PM-AMPase and GA-AMPase, which were also present in sufficient amounts in PM and GA of peanut cotyledons, were not purified and characterized. Hence the inter-relationship between various PM-AMPases and GA-AMPases remained uninvestigated. In the present study, our attempt was to purify to homogeneity other molecular forms of the PM-AMPase and GA-AMPase and study their immunological characteristics to know more about the inter-relationship of the enzymes. Our approach has been following:

(i) To prepare high specific activity, highly enriched, GA and PM fractions, free from cross contamination.

(ii) Selective solubilization of the enzymes using a suitable detergent.

(iii) Purification of the enzyme to apparent homogeneity and preparation of polyclonal monospecific antibodies.

The scheme used for the preparation of subcellular membrane fractions (Sharma et al., 1986) yielded highly enriched PM and GA fractions from peanut cotyledons, which were essentially free from cross contamination. Various detergents have been used to convert membrane-bound enzyme to a soluble one (Hjerten et al., 1988; Mittal et al., 1988; Turnay et al., 1992). We found CHAPS to be an efficient detergent in selective solubilization of the AMPase activity from PM and GA fractions (Table 4.2). Substantial purification was also achieved through CHAPS solubilization (Tables 4.2 and 4.7). The efficiency of solubilization was comparable with that of the octylglucoside used in previous studies (Mittal et al., 1988). In addition, gel filtration and DEAE-cellulose column chromatography results showed that, unlike octylglucoside, the CHAPS preferentially solubilized only one form of the enzyme, which appeared to be corresponding to a less dominant form of the two enzyme peaks obtained from the octylglucoside extract. The reason for this could be that various solubilization procedures convert membrane-bound enzymes to solubilized ones with different catalytic and physiochemical properties (Colemann, 1973). It seems that in the present case the orientation of the low molecular form of PM-AMPase was such which made it a favourable target for the detergent molecule when the detergent interacted with the membrane proteins. Another deviation in the purification scheme adopted from the previous work was the introduction of the gel filtration step before DEAE-cellulose column chromatography. Thus, using a modification in the purification scheme, by the way of change in detergent and introduction of additional step of gel filtration, resulted in homogeneous preparation of both, PM-AMPase and GA-AMPase, as indicated by the SDS-PAGE and Western blots. Since, GA-AMPase and PM-AMPase were purified to homogeneity by the same protocol, both enzymes were exposed to identical environ-

ment during purification. Therefore, a comparable change in natural environment, which the membranes provided to the enzyme, would be expected in both the cases.

Generally, detergent treatment destroys the hydrophobic environment of membrane structure releasing proteins and the hydrophobic constituents of the membrane, such as phospholipids, into hydrophilic medium. A number of membrane-bound enzymes are known which are no longer functional outside their natural environment (Hanahan, 1984). Therefore, the purification of such enzymes in active form has been hampered by their apparent instability after detergent solubilization (Welpy et al., 1987). Fortunately, this change in natural environment, in which the enzyme was located in the cell or in isolated PM and GA fractions, did not render the enzyme inactive. Obviously, some stabilizing factors, most probably phospholipids, were released from the membrane along with the enzyme in the aqueous medium. There is extensive evidence to indicate the importance of membrane phospholipid environment on regulation and function of membrane associated enzymes. Phospholipids may act as modulators of enzymatic reactions in addition to their role as obligatory co-factors for some membrane-bound enzymes (Sandermann, 1978). There are reports that the activity of some 5'-nucleotidases is affected by the lipid environment (Houslay and Plamer, 1978; Dipple and Houslay, 1978) while other 5'-nucleotidases are insensitive to changes in properties of the lipid region, though their activity might be modulated only by a specific phospholipid component closely associated with the enzyme (French et al., 1983). In our studies, both the treatment of membrane with detergent and purification on Sephadex G-150 gel column chromatography resulted in the release of active enzyme. In fact, the solubilized enzyme had higher specific activity as compared to membrane-bound enzyme. Therefore, it can be assumed that the enzyme maintained active conformation and factors like phospholipids were available to the enzyme in the form of protein-lipid complex. On the other hand, the purified enzyme obtained from DEAE-

cellulose column chromatography was extremely labile. This was in agreement with earlier findings of Sharma et al. (1986) Mittal et al. (1988). The labile nature might be due to the removal of the stabilizing factor(s). The enzyme, however, retained sufficient activity as compared to the specific activity of PM-bound enzyme. This suggests that the phospholipids may not be directly required for the enzyme activity as such, but may be essential for maintaining the enzyme in proper orientation and thereby, preventing the denaturation of the enzyme, as has been reported in the case of thyroid-5'nucleotidase (Niedzcoiecka and Jaroszecoicy, 1990). It may be mentioned here that Windell and Unkeless (1968) reported that the enzyme purified from rat liver PM and microsomal fractions was linked with sphingomyelin. However, the data on the effect of the presence of phospholipids on purified enzyme and the functional role of the lipid component is highly inconsistent (Evans and Gurd, 1974; Nakamura, 1976; Dornand et al., 1978). This inconsistency may be due to the variation in the stability of protein-lipid complex in aqueous medium, especially in the presence of detergent. In other words, when the complex is weak, it gets dissociated and removed by ion- exchange chromatography, which seems to be the case with peanut cotyledon PM-AMPase and GA-AMPase. Nevertheless, the significant role of phospholipids in stabilizing the purified PM-AMPase and GA-AMPase is not controversial (Heidemann et al., 1985). This also explains the reason for low recovery of the enzyme activity from DEAE-cellulose column chromatography. So far, nothing is known about the topographical organization of 5'-nucleotidase within the phospholipid layer. This enzyme is localized predominantly in the external face of the membrane (Greger and Fabianowska, 1979) where neutral and positively charged lipids are concentrated (Boggs, 1980). *In vitro* addition of phosphatidylcholine to the inactive purified enzyme restored nearly 46% of the enzyme activity. These data were interpreted to mean that the specific protein-lipid interaction would have influenced the conformation of the protein, resulting in biologically active protein structures (Slavik et al., 1977). The increase in the enzyme

activity could be due to the increase in the lipid fluidity of the enzyme's immediate environment. This, in turn, would relieve a constraint on the protein molecule to increase conformational flexibility and the activity of the enzyme. Similar observations are reported in case of other 5'-nucleotidases (Pieri et al., 1991).

Glycerol was found to effectively protect the enzyme from inactivation during storage. The mechanism of protection of enzyme activity by glycerol is not known, but the presence of glycerol may be helpful in reducing the number of water molecules interacting with the enzyme and consequently in providing a more acceptable environment to the enzyme.

The 26.3Kd PM-AMPase is a glycoprotein containing almost 37% carbohydrate. Thus, the enzyme confirms the general nature of the plant 5'-nucleotidases and acid phosphatases which have varying degree of carbohydrate content (Fuzimoto et al., 1980; Rossi et al.,1981; Churchill and Sze, 1983; Kamenan and Diopoh, 1983; Ferens and Morawiecka, 1985; Basba et al., 1987; Teno et al., 1987). In the present study the carbohydrate content of the PM-AMPase was found to be lower than that reported by Sharma et al. (1986). The endo-H treatment released 90% of the total carbohydrate indicating that the oligosaccharide moiety was N-linked high mannose type. Whether the remaining 10% of the carbohydrate represents a complex type structure or not is yet to be known. Since glycosylation is restricted to the hydrophilic surface of the protein, it is likely that the structure of glycosidic moieties of these membrane-bound enzymes might influence the protein conformation, catalytic activity and protection against proteases.

The low molecular mass PM-AMPase (M_r 26.3Kd), purified from the PM of peanut cotyledons, appears to be a different protein as compared to both, the high molecular

mass PM-AMPase (M_r 55.0Kd) reported by Sharma et al. (1986) and to *Zea mays* microsomal enzyme (M_r 49.5Kd) reported by Carter and Tipton (1986). Since the molecular masses of the two enzymes were determined by SDS-PAGE under fully-denaturing conditions, the possibility of 26.3Kd protein (low molecular mass PM-AMPase) being the subunit of 55.0Kd protein (high molecular mass PM-AMPase) may be ruled out. In other words, the PM-AMPases are single polypeptide chains. However, at the moment the possibility of the presence of two or more subunits of exactly same size or the presence of same polypeptide subunits can not be ruled out.

The 26.3Kd PM-AMPase, like the 55Kd PM-AMPase, is highly specific for 5'-AMP and shows little hydrolytic activity towards other nucleotides and phosphorylated sugars as well as p-nitrophenyl phosphate. In this respect, the 26.3 Kd protein purified from the PM of peanut cotyledons, is very different from the *Zea mays* microsomal AMPase (Carter and Tipton, 1986) and AMPases from other sources, including plants which exhibit sufficient hydrolytic activity towards other nucleotides and p-nitrophenyl phosphate, in particular (Sullivan and Alpers, 1971; Evans and Gurd, 1974; Polya, 1974 and 1975). Since the *Zea mays* enzyme was purified from whole microsomal fraction, its exact subcellular location can not be ascertained and, therefore, the difference in the properties is not unexpected. Here it may be pointed out that the *Zea mays* enzyme and peanut enzyme also exhibit some striking similarities in the properties like the optimum pH, substrate specificity and in not being a zinc-containing metalloprotein.

Various immunological studies have been performed (Harb et al., 1985; Heidemann et al. 1985) to comprehend about the species specific and tissue specific nature of 5'-nucleotidase. Not much work has been done in this direction in case of plant tissues. In contrast to the previous studies, where antibodies against bovine tissue 5'-nucleotidase have been found to recognize 5'-nucleotidase from human, mouse and chicken origin,

the present studies show that the low molecular mass PM-AMPase antibodies do not recognize the 5'-nucleotidase from other plant sources. This may, therefore, reflect the existence of species specific form of enzyme.

The GA-AMPase (M_r 52.4Kd) which appeared to be closely related to the high molecular mass PM-AMPase (M_r55.0 Kd) (Sharma et al., 1986) has been found to be different from the low moleular mass (26.3 Kd) PM-AMPase. On CHAPS solubilization, 2.3-fold purification of GA-AMPase was obtained which was much lower as compared to 5-fold purification of low molecular mass PM-AMPase solubilized under similar conditions. This is probably due to the difference in the binding specificities of the two enzymes for hydrophobic compounds which may be related to different lipidic environment. Although PM-AMPase and GA-AMPase had comparable optimum pH, phospholipid effect and carbohydrate content, a marked difference in K_m and substrate specificity was observed. The GA-AMPase was found to resemble 5'-nucleotidase from *Micrococcus varians subsp. halophilus* in substrate specificity which hydrolyzed 5'-monophosphates with preference for 5'-AMP (Onishi et at., 1984). In addition to various differences described above, the difference in low molecular mass PM-AMPases and GA-AMPase was further confirmed by immunological response. Immuoprecipitation, Western blotting and double immunodiffusion studies showed that the polyclonal monospecific antibodies raised against the purified 26.3 Kd PM-AMPase did not cross react with purified GA-AMPase. In other words, two enzymes do not share the common determinants. These results also suggest that PM-AMPase and GA-AMPase are the product of different genes.

Various studies have shown the presence of glycolipid linker glycosyl phosphatidylinositol for membrane anchoring of membrane-bound 5'-nucleotidases (Stochaj et al., 1989; Bailyes et al., 1990). The soluble forms of 5'-nucleotidase were

found to be devoid of this anchor. We have not studied the membrane anchoring of PM-AMPase and GA-AMPase. However, the significant difference in the properties of two enzymes raises a question as to whether the two enzymes differ at genetic level as well ? May be the two enzymes differ in linkage to the membrane.

The generally accepted theory for the transport of proteins to the PM is that the proteins destined for export are synthesized on the RER vectorially discharged into the lumen of the RER, translocated to the GA stacks and finally are packaged in secretory vesicles for transport to PM (Palade, 1975). This occurs by a default mechanism and requires no signal . In other words, these proteins will not be retained in ER or GA unless they contain a specific signal for retention. Thus, in the present case we have a 26.3Kd glycopeptide (PM-AMPase) which is not found in GA but is localized in PM. This protein obviously travels from ER to the PM without being retained in GA by default mechanism. On the other hand, the 52.4 Kd glycopeptide (GA-AMPase) is localized in GA. As polyclonal antibodies raised against the PM-AMPase (M_r 26.3Kd) did not cross react with GA-AMPase, it indicates that this protein was retained in GA and may, therefore, contain a signal for its retention in GA. In some cases, modifications of the oligosaccharide direct the glycoprotein to its cellular location (Roth, 1987). Whether the same is true for the PM-AMPase and GA-AMPase, is not known. Glycosylation is not needed for intracellular transport of phytohemagglutinin in developing *Phaseolus vulgaris* cotyledons and for the maintenance of its biological activity (Bollini, et.al. 1985). However, the carbohydrate contents of the PM and GA-AMPase are significantly different. Further studies would be required to clarify this point. The conceptual model depicting the transport of PM-AMPase and GA-AMPase to different cellular locations is shown in Fig. 5.1.

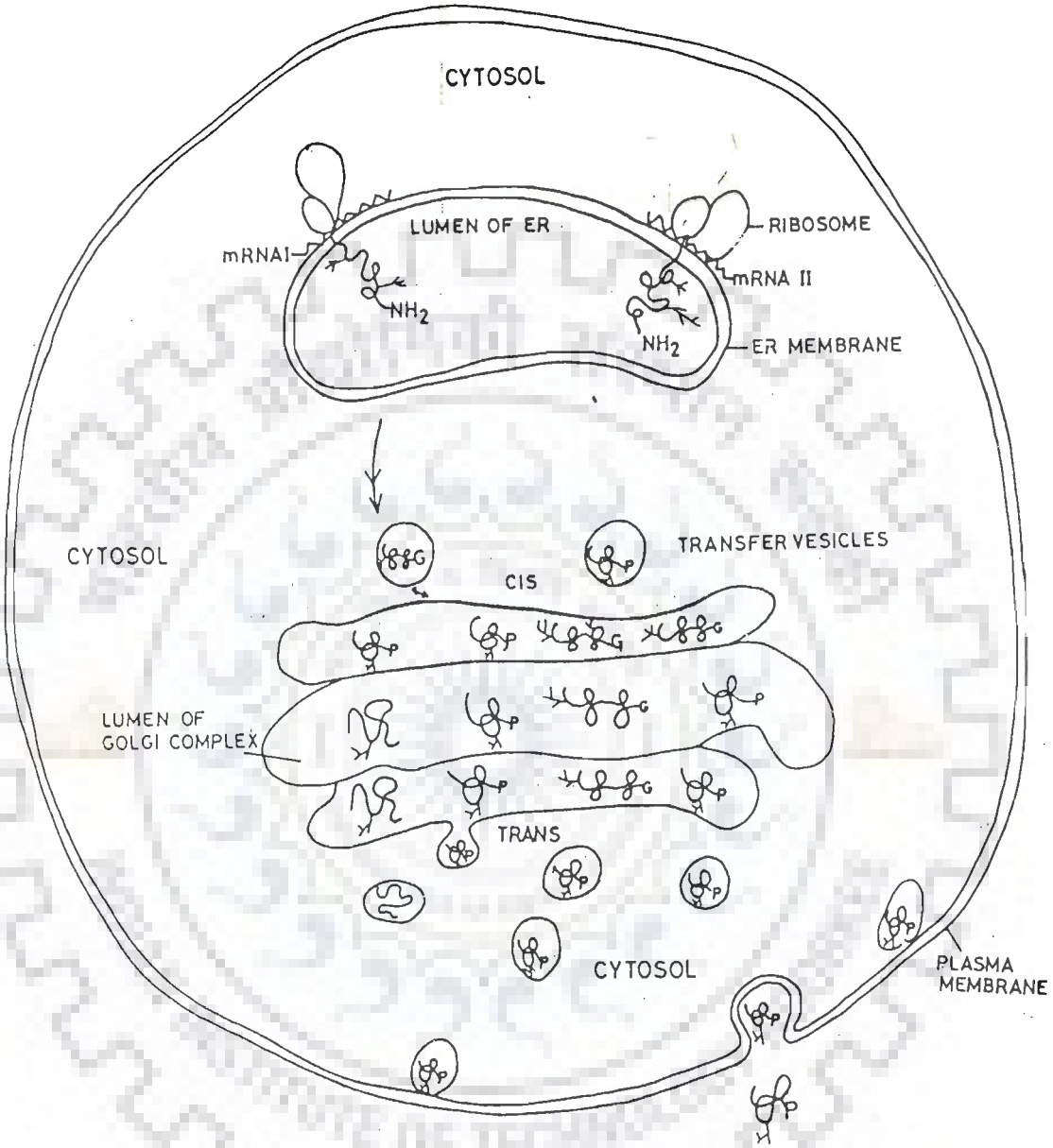


Fig 5.1 PROPOSED MODEL FOR TRANSLOCATION OF PM-AMPase AND GA-AMPase TO DIFFERENT CELLULAR LOCATIONS

P represents PM-AMPase enzyme

G represents GA-AMPase enzyme

Chapter 6

6.0 SUMMARY AND CONCLUSIONS

Two distinct molecular forms of 5'-AMPase, one from the plasma membrane (PM) and other from the Golgi apparatus (GA) membrane fractions of germinating peanut *(Arachis hypogaea L.)* cotyledons have been purified to apparent homogeneity as evidenced by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Western blot patterns. The purification protocol involved preparation of high specific activity PM and GA fractions (free from cross contamination as indicated by the presence / absence of marker enzymes for the PM and the GA fractions), selective solubilization of the enzymes from the membrane fractions, PM and GA, with 1.0% CHAPS at a protein-to-detergent ratio of 2:3, followed by gel filtration on Sephadex G-150 and DEAE-cellulose column chromatography. The purified PM-AMPase showed a molecular mass of approximately 26,300 daltons on SDS-PAGE. On native PAGE (under non-denaturing conditions) the purified PM-AMPase migrated as a tight single protein band with high electrophoretic mobility of 0.74, with respect to bromophenol blue. This shows the absence of a multisubunit structure. The PM-AMPase has pH optimum in the range of 5.0 to 5.5. K_m for 5'-AMP as substrate is 0.58 mM and V_{max} is 5.9 μ mole Pi/min/mg-protein. The enzyme is highly specific for 5'-AMP, with no significant hydrolysis of p-nitrophenyl phosphate, sugar phosphates and other nucleotides. The PM-AMPase is a glycoprotein containing approximately 37% (w/w) carbohydrate. About 90% of the carbohydrates are sensitive to endo-H (N-acetyl-β-glucosaminidase) digestion, indicating a high mannose type structure of the N-linked oligosaccharide of PM-AMPase.

The purified PM-AMPase, obtained from the DEAE-cellulose column, was extremely labile, losing its total activity within 24h at -20°C, while under similar

conditions the CHAPS-solubilized enzyme and the partially purified enzyme from Sephadex G-150 column were stable for weeks, indicating that during the ion-exchange chromatography some stabilizing factors, most likely, phospholipids, were removed. This is perhaps the reason for decline in the specific activity and low recovery of the enzyme. The presence of 20% glycerol in the elution buffer enhanced the stability of the enzyme.

Interestingly, phosphatidylcholine was found to restore about 46% activity of the denatured enzyme. This suggests that phosphatidylcholine is required for stability as well as for optimum biological activity of the PM-AMPase. In other words, the specific protein-lipid complex may provide necessary protein conformation required for the AMPase activity.

The polyclonal monospecific antibodies raised against the purified PM-AMPase did not cross react with the PM-AMPase from other plant sources (pea, chickpea, soybean and wheat) as shown by the negative response on immuno-inhibition, double immunodiffusion and Western blots. These results suggest the species specific nature of the PM-AMPase.

GA-AMPase was purified to homogeneity from the GA membrane following the same protocol as was used for the purification of PM-AMPase. The molecular mass of the GA-AMPase was found to be 52,400 daltons by SDS-PAGE. The GA-AMPase on native PAGE, under completely non-denaturing conditions, showed a single protein band with electrophoretic mobility of 0.43 which is much lower than the electrophoretic mobility of PM-AMPase (0.74). These data also suggest that like PM-AMPase the GA-AMPase is not a multisubunit structure and the two enzymes represent two distinct molecular species, perhaps the products of two different genes.

GA-AMPase has been found to be a glycoprotein containing 39% (w/w) carbohydrate. The enzyme is specific for 5'-AMP, but unlike PM-AMPase, it catalyzes

the hydrolysis of 5'-GMP, 5'-UMP and p-nitrophenyl phosphate (p-NPP) as well to a significant extent. Thus, GA-AMPase shows broader specificity than the PM-AMPase. The K_m and V_{max} values for 5'-AMP as substrate were 1mM and 6.8 μ mole Pi/min/mg-protein.

The purified GA-AMPase was highly unstable after the DEAE-cellulose column, but was stabilized to a great extent when stored in 20-30% glycerol and also required phosphatidylcholine for optimum activity. In these respects the PM-AMPase resembles the GA-AMPase.

The most striking difference between the PM-AMPase and the GA-AMPase is in their immunological responses. The GA-AMPase did not show any cross reactivity with the polyclonal monospecific antibodies raised in rabbits against the purified PM-AMPase as indicated by immunoprecipitation, double immunodiffusion and Western blots. Thus, the two enzymes show different subcellular immunological specificity. This clearly indicates that they are immunologically different protein molecules having no common antigenic determinants. These results, together with the molecular mass derived by SDS-PAGE and native gel electrophoresis, indicate that the PM-AMPase and GA-AMPase are not structurally related and represent two distinct molecular forms of the AMPase. These data are also interpreted to mean that while the GA-AMPase is retained in the GA, the PM-AMPase migrates to the PM from the ER by a default mechanism without being retained in the GA. Further studies on the relationship of the primary, secondary and tertiary structures of the two enzymes would be quite interesting for the understanding of the sorting and transport of membrane glycoproteins in plants.

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