STUDIES ON PLASMA MEMBRANE 1,3-β-D-GLUCAN SYNTHASE OF PEANUT COTYLEDON CELLS

A THESIS

submitted in fulfilment of the
requirements for the award of the degree
of
DOCTOR OF PHILOSOPHY

By

UJJWALA KAMAT

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

NOVEMBER, 1991

To,

"Ma"

who instilled in me the courage and confidence to face and overcome all difficulties that have ever come my way.

"Pappa"

who inculcated in me the scientific attitude and introduced me to the joy of learning.

Candidate's Declaration

I hereby certify that the work which is being presented in this thesis entitled STUDIES ON PLASMA MEMBRANE 1, 3-β-D-GLUCAN SYNTHASE OF PEANUT COTYLEDON CELLS in fulfilment of the requirements for the award of the Degree of DOCTOR OF PHILOSOPHY, submitted in the Department of Biosciences & Biotechnology of the University is an authentic record of my work carried out during a period from February, 1986 to November, 1991 under the supervision of Prof. C. B. Sharma and Dr. Vinay Sharma, Department of Biosciences and Biotechnology, University of Roorkee, Roorkee.

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

Ujjwala Kamat
(UJJWALA KAMAT)

Date ; 29.11.91

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

29.11.91
VINAY SHARMA
Lecturer,
Dept. of Biosciences & Biotechnology

C. B. Sharma
C.B. SHARMA 29.11.91
Professor,
Dept. of Biosciences & Biotechnology

The Ph.D. Viva-Voce examination of Miss. Ujjwala Kamat

Research scholar has been held on April 10, 1992

C. B. Sharma

10.4.92

10.4.92
Signature of Guide(s)

Signature of External Examiner

ABSTRACT

1,3-β-D-glucan synthase (UDP-glucose : 1,3-β-D-glucan 3-β -D-glucosyltransferase, EC 2.4.1.34) is a plasma membrane localized enzyme which catalyzes the synthesis of callose (1,3- β-D-glucan) from UDP-glucose in higher plants. In this study two forms of 1,3-β-D-glucan synthase (GS-IIA and GS-IIB) have been separated from a plasma membrane fraction from the 7-day old germinating peanut cotyledons using selective solubilization of the enzymes with 0.5% digitonin at a protein-to-detergent ratio of 1:6, sucrose density gradient centrifugation and chromatography on hydroxylapatite column. Both forms were subsequently purified to apparent homogeneity by chromatography on hydroxylapatite (second column) followed by chromatography on DEAE-sephadex A-50. The specific activity of the purified GS-IIA and GS-IIB was increased about 636- and 64-fold, respectively, relative to the crude microsomal membrane fraction. On sodium dodecylsulfate polyacrylamide gel electrophoresis GS-IIA and GS- IIB migrated as a single protein band with molecular masses of 48K and 57K, respectively. Determination of the H_2N-terminal amino acid by Edman degradation gave phenylthiohydantoin derivatives of L-leucine for GS-IIA and L-lysine for GS-IIB, indicating the presence of single polypeptides in both enzyme forms.

The purified GS-IIA and GS-IIB were found to be quite specific for UDP-glucose as the glucosyl donor and required Ca^{2+}, at an optimum concentration of 2-5mM, for activity. The activity of both enzymes was inhibited by nucleotides (ATP, GTP, CTP, UDP and UMP). The enzyme activity was also inhibited by the addition of

EDTA or EGTA to the enzyme, but this inhibition was fully reversible by the adition of Ca^{2+}. The reaction product formed during incubation of UDP-[^{14}C] glucose and cellobiose with the purified enzymes was susceptible to digestion by exo-(1,3)-β-D-glucanase, but was resistant to α- and β-amylases and to periodate oxidation, indicating that polymer formed was 1,3-β-D-glucan and β-1,4 and β-1, 6 linkages were absent.

Polyclonal monospecific antibodies against GS-IIA and GS-IIB were raised in rabbits. The immune serum obtained with GS-IIA inhibited the enzyme activity and reacted specifically with GS-IIA on immunodiffusion plates. The GS-IIA immune serum also did not cross-react with GS-IIB. These results further confirmed the homogeneity of the GS-IIA preparation. The immune serum made with GS-IIB was found to be completely inactive against both GS-IIB and GS-IIA, indicating that the two enzyme forms were immunologically different.

ACKNOWLEDGEMENT

This is an oppurtunity for me to convey my gratitude towards those who helped in this most coveted of academic endeavor. When I think of the efforts, and the patience that was needed during this gruelling period, the good-will that prodded me at every faltering step, quietly slips into my mind and leads it down memory-lane to recollect and rejoice in human magnanimity. Here is an humble attempt to acknowledge the encouragement and assistance that moulded this accomplishment.

It would be difficult and almost impossible to achieve excellence without the blessings and good-wishes of our elders. Culturally and traditionally one imbibes that, in an academic institution the teacher is the most respectable elder and is worthy of the highest esteem. With such resounding echoes I, with sincerety, thank my guides; **Dr. C.B. Sharma**, Professor and Head, and **Dr. Vinay Sharma**, Lecturer, Department of Biosciences and Biotechnology, University of Roorkee, Roorkee, for their constant support and advice on the designing and execution of the planned approach to this research problem.

I will always with the deepest regards remember my supervisor and mentor, **Prof. C.B. Sharma** who reflected on me the importance of work-discipline and always encouraged me to reach much above my natural abilities. His consideration and patience during my difficult phases have always helped me persist. I am extremely fortunate to have been trained and initiated into the Scientific-world under his able guidance. His total dedication to science and the pursuit of knowledge will forever remain a source of

inspiration to me.

I am grateful to all the other faculty members of the Department for the co-operation extended during this period of study.

Life is a treasure which seems more precious when wonderful people brighten it. Over these years there have been, some who were always with me, some who flashed by and some others sauntered in with imposing reassurance.

I applaud **Ms. Sulekha** and **Ms. Veena** for creating a healthy camaradarie which helped me sail through tough times unscathed. Their undettering support will never be forgotten. I appreciate and am grateful to my colleagues; **Dr. Kush Garg** for the immense and valuable help readily rendered at different stages of this work and **Mr. Rajeev Gautam** for his kind and quick assistance.

I am particularly thankful to **Ms. Sagarika** and **Ms. Nirupama** for their affectionate caring and was lucky to be amidst the exuberance and vivacity of **Nandana, Glory** and **Swagata** which always invigorated me.

The congenial and friendly atmosphere in the laboratory maintained by **Ritushree** and other colleagues made work enjoyable and peaceful.

Ma, Pappa, Santosh and **Hemant** have been like pillars of strength during these years and words cannot account their support.

My sincere thanks to **Mr. Nirmal Kumar** for meticulously typing this manuscript, to **Mr. Rajiv Bansal** for preparing neat drawings

and to **Mr. Mohan Sharma** for good photography.

I duly acknowledge the financial assistance provided to me by the **Council of Scientific and Industrial Research (CSIR), India.**

Ujjwala Kamat

(UJJWALA KAMAT)

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADP: - Adenosine 5' - diphosphate

AMP: - Adenosine 5' - monophosphate

ATP: - Adenosine 5' - triphosphate

Amino acids:

L - Ala : L - Alanine

L - Asp : L - Aspartic acid

L - Gly : L - Glycine

L - Try : L - Tryptophan

L - Val : L - Valine

L - Glu : L - Glutamic acid

L - Leu : L - Leucine

L - Met : L - Methionine

L - Ileu : L - Isoleucine

L - Pro : L - Proline

L - Thr : L - Threonine

L - Tyr : L - Tyrosine

L - Phe : L - Phenylalanine

L - Ser : L - Serine

L - Lys : L - Lysine

^{o}C - ^{o} Centigrade

Ci/mol - curie/mole

cm - centimeter

conc - concentration

CHAPS - (3- [(3 - cholamidopropyl)-dimethylammonio] -1 propane-sulphonate)

cpm - counts per minute

CTP - Cytosine 5' - triphosphate

DEAE	-	Diethylaminoethyl-sephadex ; cellulose
dol	-	Dolichol
EDTA	-	Ethylene diamine tetraacetic acid
EGTA	-	Ethylene glycol bis (2-aminoethyl ether) N,N,N',N' - tetraacetate
ER	-	Endoplasmic reticulum
fig	-	Figure
g	-	gram
GA	-	Golgi apparatus
GDP	-	Guanosine 5' - diphophate
GMP	-	Guanosine 5' - monophosphate
GTP	-	Guanosine 5' - triphosphate
h	-	hour
K	-	kilo dalton
Ki	-	Inhibitor constant
Km	-	Michaelis-Menten constant
log	-	logarithm
M	-	Molar
mA	-	milliampere
mg	-	milligram
min	-	minute
µg	-	microgram
µl	-	microlitre
µci	-	microcurie
µM	-	micromolar
ml	-	millilitre
mM	-	millimolar
nm	-	nanometer

nmoles	-	nanomoles
PPO	-	Polyphenoloxidase
PTC	-	Phenylisothiocyanate
PTH	-	Phenylthiohydantoin
SDS	-	Sodium dodecyl sulphate
sec	-	second
TEMED	-	N,N,N' N'-tetramethylethylene diamine
UDP	-	Uridine 5' - diphosphate
UMP	-	Uridine 5' - monophosphate
v/v	-	volume/volume
Vmax	-	maximum velocity attained by a enzyme catalyzed reaction.
w/w	-	weight/weight
w/v	-	weight/volume.

1.0 INTRODUCTION.

In recent years understanding of the biosynthesis of cell wall molecular components has attracted considerable interest, (Wasserman and McCarthy, 1986; Delmer, 1987; Sloan et.al., 1987). Isolated membrane fractions from a number of plants contained a 1,3-β-D-glucan (callose) synthase. This enyzyme is largely found on the plasma membrane and in most cases is latent and is activated only by perturbed conditions which leads to some loss of membrane permeability (Kauss, 1985). Callose is a polysaccharide containing a high proportion of 1,3-β-D linked glucose and is rapidly deposited in response to physical or chemical stress adjacent to plasma membrane and often at the connections that unite the protoplasm of contiguous cells, presumably to isolate the injured area (Eschrich,1975). The localized deposition and the speed of callose formation may be important for the defence against the pathogens as callose is also a major component of papillae or wall appositions which are formed at the sites of attempted penetrations by invading fungal hyphae (Aist,1976; Bell,1981). Callose is similarly found around lesions in certain virus-infected plants where it may help to prevent the spreading of the virus (Shimomura and Dukstra, 1975; Bell, 1981). In addition callose is generally formed as a transient wall material at sites which are altered during special development steps such as pollen maturation and sieve-pore formation (Fincher and Stone, 1981), in incompatible-matings (Anderson et al., 1983) and gravitropism (Jaffe and Leopold, 1984) All these processes require that callose synthesis is a well regulated process. Inspite of the fact that callose seems

to play a vital role in these developmental processes the enzyme 1,3-β-D-glucan synthase, also known as glucan synthase-II which catalyzes the synthesis of callose in plants has not been purified to homogeneity so far and the information on the regulation of cell wall polysaccharide synthesis has been extremely inadequate. In order to understand the mechanism and the molecular basis of the action of callose in various biological processes and especially its role in the defence mechanism against pathogen attack it seemed to us, essential to purify this enzyme to homogeneity. This study will also provide an insight into the hypothesis which assumes that the callose synthase is an altered form of cellulose synthase. (Jacob and Northcote, 1985; Delmer, 1987). In this study we have chosen peanut cotyledon as the source of enzyme because its an important crop in India and also the seeds are large enough to prepare sufficient amount of purified plasma membrane fractions.

The main objectives of the research described in the thesis were as follows :

1. **To prepare highly enriched plasma membrane fraction from peanut cotyledon of appropriate physiological state.**

2. **To solubilize the plasma membrane-bound enzyme with detergents in active and stable form suitable for purification.**

3. **To purify to homogeneity and characterize the 1,3-β-D-glucan synthase.**

2.0 LITERATURE REVIEW

2.1 SYNTHESIS AND DEPOSITION OF PLANT GLUCANS

The discovery of UDP-glucose by Leloir (1951) changed the ideas of complex polysaccharide biosynthesis. UDP- glucose was demonstrated to be a direct precursor *in vitro* for the biosynthesis of trehalose, sucrose, starch, callose and microbial cellulose (Leloir, 1964). Glucose containing sugar nucleotides, which were considered as the natural substrates for cellulose formation, have been found in different plants. UDP-glucose has been detected in growing *Phaseolus aureus* tissue (Solms and Hassid, 1957), in bamboo tissue (Su, 1965), in wheat and oat seedling (Elnaghy and Nordin, 1966), in developing cotton fibres, (Franz, 1969), in cambial tissue (Cumming, 1970), in suspension-cultured sycamore cells (Brown and Short, 1969) and in a great variety of other plant materials.

The bicsynthesis of cellulose in a cell-free system was first demonstrated by Glaser (1958) using a particulate enzyme preparation from *Acetobacter xylinum*. Glucose residues were transferred from the substrate UDP-glucose to an alkali insoluble polysaccharide identified as cellulose. Barber et al. (1964) repeating the above experiments and comparing the substrate specificity of different NDP-glucoses, were able to demonstrate that only UDP-glucose, and to a much lesser extent TDP-glucose could serve as glucosyl donors for cellulose biosynthesis. ADP-glucose and CDP-glucose were completely ineffective. Using a similar system from higher plants (*Phaseolus aureus*) Feingold et al. (1958) obtained a 1-3-β-

glucan using UDP-glucose as substrate. Ordin and Hall (1967) confirmed the hypothesis put forward by Brummond and Gibbons (1964; 1965) that a particulate enzyme preparation from Lupinus albus is capable of catalyzing the incorporation of [^{14}C] D-glucose from UDP [^{14}C]-D-glucose as well as GDP [^{14}C]-D-glucose. These workers also found that the polysaccharide formed from GDP-D-glucose with Avena sativa enzymes consisted exclusively of β-(1,4) linkages, and the hydrolysate of the material formed from UDP-glucose contained β-(1,3) glucosidic linkages as well. One of the more important findings in recent years was that concerning the influence of substrate concentration upon the type of glycosidic linkage formed in vitro. High levels of UDP-glucose (mM) preferentially led to the production of 1,3-β-linkages whereas with low levels (μM) 1,4-β-linkages resulted (Peaud-Leonel and Axelos, 1970; Smith and Stone, 1973; Van der Woude et al., 1974).

2.2 PLANT GLUCAN SYNTHASES

The synthetases for cell wall polysaccharides are invariably membrane-bound and presumably could exist as a complex which by concerted action, might control the proportion and sequence of residues, linkage types or branches. Thus for the (1-3); (1-4)-β glucan the proportion of (1-3) and (1-4) linkages could be controlled by two linkage specific synthetases with different affinities for the terminal saccharide units. Infact oat coleoptile membrane preparation, which can synthesize (1-3); (1-4) β- glucans in addition to (1-3) and (1-4) β-glucans, can be fractionated to yield separate (1-3) and (1-4) β-glucan

synthetases (Tsai and Hassid, 1973), but whether in combination these enzymes synthesize a (1-3); (1-4) β- glucan or whether a specific (1-3); (1-4) β-glucan synthetase exists is not known. An alternative possibility is that the heteropolymers are assembled from preformed blocks. Thus Brett and Northcote (1975) suggested that in the synthesis of mixed-linked polysaccharide, oligosaccharides containing a single linkage type might be first assembled on a lipid or protein intermediate and subsequently formed by a second linkage to form the completed polysaccharide.

2.2.1 1,3-β-glucan synthase

Plant coated vesicles have been shown to possess a glucan synthase, an enzyme that is certainly not accepted to occur in animal coated vesicles. Griffing et al. (1986) provided evidence for the presence of (1,4)-β-D-glucan synthase (Glucan synthase-I) activity in coated vesicles isolated from soybean protoplasts. Since this enzyme is usually regarded as a marker for Golgi membranes the authors concluded that, although not carrying exportable wall polysaccharides, the coated vesicles represent instead a transport vehicle for precursor 1,3-β-D-glucan synthase (Glucan synthase-II) which is found in the plasma membrane.

Robinson et al. (1987) and Robinson and Depta (1988) have confirmed that a glucan synthase activity is present in coated vesicles isolated from zucchini hypocotyl tissue. However, the activity was that of glucan synthase-II (GS-II) rather than that of glucan synthase-I (GS-I). They showed that the product formed by coated vesicles is a 1,3-β-glucan and is in the form of short, needle-like microfibrils. Besides forming larger amounts

of the ethanol insoluble product as previously recorded for glucosyl transferases, it also was stimulated several folds by Ca^{2+} ions and spermine. Thus, it could be possible that the coated vesicles transport a cryptic form of the enzyme GS-II to the PM. Coated vesicles are abundant in plants, particularly in regions of active cell wall synthesis. They are an important part of the traffic between the Golgi compartment and the plasma membrane and the lysosome in animal cells.

The product of GS-II is a β-1-3 linked glucan, callose (Heiniger and Delmer, 1977; Raymond et al., 1978; Henry et al., 1983). Soybean cell - suspension cultures have no callose in the cell wall, but it may arise when the cultures are mechanically wounded. Hanke and Northcote (1974); Brett (1978); found no callose produced by soybean protoplasts. However, methylation analysis of the radioactivity labeled, newly synthesized cell wall and extracellular material secreted from soybean protoplasts show extensive incorporation into 3-linked glucose, indicating the presence of callose (Klein et al., 1981).

Langerbartels et al. (1981) correlate the activities of GS-I and GS-II with the ability of carrot protoplasts to form a new cell wall. Although GS-II activity remains relatively constant during wall regeneration (a little higher in freshly isolated protoplasts), GS-I activity increases at the time cells begin to incorporate substantial amounts of glucose into the cell wall. Hence, Langebartels et al. (1981) suggest that GS-II is a wound enzyme stimulated by the protoplasting process and GS-I is active in wall regeneration.

The role of coated vesicles in cell wall biogenesis is explained by the increase in activity of GS-I which is an important enzyme. If GS-I participates in xyloglucan synthesis (Hayashi and Matsuda, 1981) or integration, the coated vesicles may act to sort GS-I activity among the Golgi internal in order to organize the complex process of wall assembly. Alternatively, if GS-I is a cellulose synthase proenzyme, coated vesicles coming from or going to the plasma membrane may be recycling or repositing the cellulose synthase (Shore et al., 1975; Hayashi and Matsuda, 1981). If coated vesicles are recycling plasma membrane, they must be doing so selectively, since they seldom contain marked GS-II activity.

2.2.1.1 Effectors of GS-II

2.2.1.2 Effect of Ca^{2+}

Calcium ions at low concentrations stimulate GS-II activity (Kauss et al., 1983). However, earlier workers (Eschrich, 1975), thought that Ca^{2+} ions are necessary for the deposition of callose. Nevertheless, there is now sufficient experimental evidence available which shows that Ca^{2+} ions are direct effectors of the enzyme and do not act with the help of an auxiliary enzyme system such as phosphorylation or dephosphorylation (Kauss et al., 1983).

Also very low concentration of free calcium ions (10^{-7}M) in the cytoplasm of giant algal cells and in plant protoplasts have been reported by Williamson (1981) and Gilroy et al. (1986). There is yet another report which says that the free Ca^{2+} concentration in the cytoplasm of animal and plant cells is held

at a low level, between 10^{-7} to 10^{-8} M. (Cheung, 1980).

The GS-II assay systems containing 5mM Mg^{2+} a 10-15 fold activation by Ca^{2+} was found at saturating levels of Ca^{2+} (50 to 100 μM). In the absence of Ca^{2+}, 5 mM Mg^{2+} also slightly activated the enzyme. If Mg^{2+} is omitted altogether then there is a 40 to 50-fold activation, indicating that Mg^{2+} ions cannot replace Ca^{2+} as far as activation of GS-II is concerned. Also, it is thought that the interaction of Ca^{2+} with the enzyme is a direct allosteric type which leads to activation of enzyme and not a phosphorylating/dephosphorylating one (Kauss, 1986). Also, Delmer et al. (1977) observed that in the absence of Ca^{2+} the cotton fibre GS-II had a low affinity for UDP-glucose.

Kauss and Jeblick (1985) have reported that Ca^{2+} appears to interact at a different site on the 1,3-β-D-glucan synthase than the interaction site of polyamines like poly-L-lysine, poly-L-ornithine and ruthenium red. This site specificty of Ca^{2+} was confirmed by using La^{3+}, which is known to be specific for many Ca^{2+} binding sites and can very effectively inhibit the activation by Ca^{2+} but could not affect the binding of the polyamines. Girard and Maclachlan (1987) have shown an enhancement of the GS-II activity in pea membranes by the addition of Ca^{2+}, half maximum stimulation with added Ca^{2+} was observed at about 75 μM. The role of Mg^{2+} ions in GS-II activation with respect to Ca^{2+} is still uncertain. For example, Hayashi et al. (1987) reported that the presence of Mg^{2+} seems to cause some co-operative conformational change and/or aggregation of the enzyme and also results in synthesis of a more insoluble

aggregated form of glucan. Paliyath and Poovaiah (1988) have proposed a regulatory model for β-glucan synthase. In this model the promotion of GS-II activity by Ca^{2+} and by phosphorylation involving Ca^{2+} and calmodulin has been suggested.

Sodium fluoride stimulates the activity of C. albicans GS-II independently of the activator GTP, an effect which is additional to the possible protection of GTP from degradation by phosphates by NaF. (Orlean and Ward, 1983).

2.2.1.3 Effect of phospholipases C,D and A2

Exposure of plasma membrane to phospholipases C,D and A2 resulted in rapid inactivation of glucan synthase. This inactivation could not be reversed by reconstituting phospholipase treated membrane with exogenous phospholipids. With the help of phospholipase treated plasma membrane and Triton X-100 treated plasma membranes many conclusions were drawn. It was found that the Triton X-100 treated membranes could be reactivated with phospholipids. These results suggest that phospholipases affect boundary lipid differently than detergent extraction. Where boundary phospholipid was removed by detergent extraction it was possible to restore enzymic activity by adding phospholipids. After exposure to phospholipases, all attempts to restore the enzyme activity were unsuccessful. One possible explanation is that various phospholipase reaction products such as lysophospholipid bind to and inhibit glucan synthase. However, if this were the sole cause of enzyme inactivation, there might be some return of activity after removal of these products. But, it was observed that activity could not be restored to phospholipase treated

glucan synthase by removing inhibitors either by centrifugation or detergent extraction followed by adding back phospholipid. It seems more likely that glucan synthase contains covalently bound phospholipid. This covalently bound phospholipid is like an anchor for membrane proteins. Also, since glucan synthase is an integral membrane protein it contains both, covalently as well as non-covalently bound boundary lipid. Thus, the critical site of action for the phospholipases is at phospholipid covalently attached to the enzyme. (Sloan and Wasserman, 1989). This will render the enzyme irreversibly inactive.

2.2.1.4 Activation by polyamines

A synergistic activation by polyamines of GS-II has also been demonstrated. For example, when spermine (a polyamine) is added along with Ca^{2+}, the effect is additive. In other words, the effect obtained is much more than is possible to obtain either by spermine or Ca^{2+} individually. Furthermore, it has been reported that much lower Ca^{2+} concentration is required for enzyme activation in the presence of spermine. Other polyamines like spermidine, putrescine and ornithine have also been shown to activate GS-II, but to a lesser extent as compared to spermine (Kauss and Jeblick, 1986).

2.2.1.5 Elicitors of callose synthesis

Kohle et al. (1985) have observed in suspension-cultured cells of Glycine max (soybean cells) that, synthesis of callose starts within twenty minutes of treatment with chitosan and the accumulation of 1,3-β-D-glucose polymer (callose) in the wall. The effect of chitosan is enhanced by polymyxin-B at low

concentrations; this antibiotic alone at higher concentrations can also induce callose synthesis. Callose synthesis is immediately stopped when external Ca^{2+} is bound by ethylene glycol bis (2-aminoethyl ether) N,N,N',N'-tetracetate (EGTA) or cation exchange beads and partly recovers upon restoration of 15 μM Ca^{2+}.

Another report by Waldmann et al. (1988), states that in suspension-cultured cells of Glycine max and Catharanthus roseus callose synthesis can be induced by digitonin and chitosan. Also, it was observed that the leakage of a limited pool of electrolytes precedes callose formation, K^+ representing the major cation lost. Poly - L - ornithine, as well as the ionophores A 23187 and ionomycin, also induce some callose synthesis, but to a lesser extent. Callaghan et al. (1988) have reported about an endogenous, heat stable activator of GS-II in mung bean (Vigna radiata). The compound has been indicated to be an alkali labile β-linked glucolipid. Reihl and Jaffe (1984) have observed that GS-II activity increases immediately when excised tendrils of pea are mechanically perturbed.

2.2.1.6 Inhibitors of GS-II

Nifedipine partly blocks callose synthesis as well as the digitonin induced increase in net Ca^{2+} uptake. The above indicates that the addition of these various substances might lead to membrane perturbation causing the common event of an increase in net Ca^{2+} uptake which results in callose deposition by a direct activation of the Ca^{2+} - dependent and plasma membrane located GS-II. Kauss and Jeblick (1986) have observed

that lysophosphatidylcholine, platelet activating factor, acylcarnitine, and echinocandin-B can also fully inhibit the enzyme. Inhibition is observed both when the enzyme is activated by Ca^{2+} or by trypsinization. At low amounts some of the substances can also cause stimulation. These effects all may result from a displacement of certain endogenous phospholipids necessary for optimal activity of the enzyme. The red beet GS-II showed a susceptibility to *in situ* inactivation by phenolic compounds just as the cytosolic enzymes. This could be due to the presence of endogenous phenolics and PPO (Mason and Wasserman, 1987).

2.2.1.7 Solubilization of the membrane-bound GS-II by detergents

Enzymes which are extrinsic or merely adsorbed or bound to membranes by divalent ions, H-bonding or electrostatic charge should be readily removed by washing with high salt concentrations and/or chelating agents. But this does not appear to apply to any of the components required for β-glucan synthase activity. There are several reports of the solubilization of glucan synthase-II from different plant sources utilizing different types of detergents. The most commonly used detergents are the zwitterionic or ampholyte detergents and the nonionic detergents. It was found by some workers that Triton X-100, Nonidet P-40, Brij and octylglucoside were detrimental to the activity of GS-II (Heiniger, 1983; Eiberger et al., 1985).

The detergents most commonly used for the solubilization of GS-II were digitonin and CHAPS. Digitonin leads to an activation in

GS-II. From 0.01 to 0.02% (w/v) digitonin there is an almost 2-fold stimulation of the activity. Higher digitonin concentrations did not result in any further stimulation. Upto 0.2% (w/v) no inhibitory effect was observed (Heiniger, 1983). But in contrast, Lawson et al. (1989) have reported a 10% stimulation at 0.1% concentration. Also at 0.5% and 1.0% digitonin, glucan synthase activity was decreased by 25% and 45% respectively.

Hayashi et al. (1987) have observed that the mung bean and cotton GS-II was stimulated at low concentrations (0.01-0.05%) of digitonin. This stimulation in the membrane preparation could be due to the unmasking of activity of outside-out vesicles. Also, it was observed that digitonin increases the Vmax but has no effect on the apparent Km for UDP-glucose.

Morrow and Lucas (1986) have also observed that in sugar beet the enzyme is membrane associated and it is stimulated by digitonin. CHAPS (3 - [(3 - cholamidopropyl) - dimethylammonio] - 1 - propane sulphonate) is also a detergent which has been widely used for the solubilization of the membrane-bound GS-II. Lawson et al. (1989) have reported considerable solubilization of the enzyme using CHAPS. With a solubilization buffer containing only 0.5% CHAPS, approximately 80% of the total activity was solubilized. Sloan et al. (1987) have discussed solubilization of a glucan synthase from red beet (Beta vulgaris L.) storage tissue. In a one-step solubilization method a single concentration of CHAPS was used. At 0.6% concentration (which is the CMC of CHAPS) more than 70% of the total activity was

solubilized from microsomes. Also an inclusion of 0.01% to 0.02% digitonin in assay mixtures was essential for activity to be expressed, with little activity seen in its absence. Neither octylglucoside or phospholipid could substitute for digitonin. In a two-step CHAPS solubilization procedure, the specific activity of glucan synthase-II was increased 5 to 6-fold compared to a 1 to 2-fold increase obtained with one-step solubilization. In the two-step solubilization, firstly, contaminating proteins are removed by treating membranes with 0.3% CHAPS in the presence of Mg^{2+}. GS- II activity is found in the pellet with 40-50% of contaminating proteins removed. In the second step the enzyme is released from the pellet with 0.6% CHAPS in the presence of 1 mM EGTA and EDTA.

2.3 CALLOSE

Callose is a 1,3-β-D-glucan polymer, the product obtained by the transfer of glucose units onto a primer by the enzyme, 1,3-β-D-glucan synthase. The nature of the primer for callose is not known, but it is very likely to be a glycoprotein, or a glucoprotein could be a plausible candidate. Possibly the same glucoprotein could also prime other forms of glucan synthesis (Stoddart, 1984).

In the case of cell wall polysaccharide biosynthesis in the higher plant system and particularly cellulose formation, it is still not clear if and how the glucan chains are initiated and the fibrillar network is formed at the plasma membrane and inserted into the cell wall. Kemp and co-workers, (Kemp and Loughman, 1973; Kemp and Loughman, 1974; Kemp et al., 1978)

reported the formation and requirement of glucosylinositol as a possible primer for 1,3-β-glucan synthesis.

UDP-glucose is the only known and proven physiological donor of the glucose moiety to the primer to form the 1,3-β-D-glucan polymer. Generally 1,3-β-glucan or callose in the algae Phaeophycea has been found to be a storage polysaccharide called laminaran. But in another algae Caulerpa simpliciuscula it was found that on wounding, the damaged surface was sealed with a plug which was a gelled form of a low molecular weight (1-3)-β-glucan and this is present in solution form in the vacuole of this algae (Percival and McDowell, 1981).

The presence of 1,3-β-glucan has also been reported to occur in the walls of all fungi with the exception of the hyphal walls of Zygomycetes (Bartnicki-Garcia, 1968; Gorin and Spencer, 1968; Rosenberger, 1976). In S. cerevisiae 1,3-β-D-Glucan is located in and around the bud scars. (Houwink and Kreger, 1953; Bacon et al., 1966; Beran et al., 1972).

In higher plants callose has been associated with reaction wood formation. 1,3-β-glucan has been extracted from reaction woods of Pinus resinosa (red pine), Pinus sylvestris (Scot's pine) and Larix larcinia (tamarack). Callose deposits have also been found in response to several types of stress imposed on a plant by changes in the environment. These include, alterations in water availability, changes in ambient temperature, increases in salinity and the presence of toxic molecules or metal ions. The mechanism of the induction of this response appears to be due to

alteration in the environment of plasma membrane bound GS-II (Fincher and Stone, 1981). A well-documented response to chemical and physical trauma is the appearance of callose, identified on the basis of its anilene blue fluorescence. These deposits have been found at the plasma membrane-wall interface, in pit fields and on sieve plates (Brett, 1978; Bell, 1981; Morrow and Lucas, 1986).

2.4 PURIFICATION OF GLUCAN SYNTHASE-II

Purification studies on this enzyme have been carried out by several workers. Wasserman and McCarthy (1986) have shown that the knowledge of this enzyme's phospholipid milieu and susceptibility to proteolysis is important both from the standpoint of its purification and physiological role. Efforts to purify the 1,3-β-D-glucan synthase of red beet storage tissue in the presence of detergents have shown that phospholipids are modulators of enzyme activity. They found there was a loss in activity when membrane was extracted with Triton X-100 and found that activity could be restored to the Triton pellet by addition of positively charged phospholipids. Partial purification of digitonin solubilized β glucan synthase from red beet root using rate zonal density gradient centrifugation gave a glucan synthase with a specific activity 40-fold greater than that found in microsomes (Eiberger and Wasserman, 1987). A polypeptide that was specifically labeled by UDP-[^{3}H]-pyridoxal with a molecular mass of 42K was identified from mung bean fractions. It was stimulated by Mg^{2+} or Ca^{2+} and it was found to co-purify with GS-II activity (Read and Delmer, 1987).

Frost et al. (1990) using the technique of photoaffinity labeling with 5-azidouridine as the probe have identified a 57K polypeptide as the UDP-glucose binding polypeptide of the 1,3-β-D-glucan synthase isolated from red beet and celery. They have indicated that the 57K represents the substrate-binding and cation-regulated component of the 1,3-β-D-glucan synthase complex of higher plants. Lawson et al. (1989) have characterized and identified 3 polypeptides associated with 1,3-β-D-glucan synthase activity from Daucus carota. The photoactivatible affinity label 5-azido-uridine 5' β-(^{32}P) diphosphate glucose was used to identify the 150, 57 and 43K polypeptides. The 57K was thought to be the same as that found in red beet and celery, which co-purifies with the 1,3-β-D-glucan synthase activity. Recently this enzyme has been purified 12-fold from suspension cells of Glycine max. using successive centrifugations on two linear sucrose gradients. Also antibodies were raised against the most purified polypeptide which was 31K and these antibodies after purification were found to specifically label a 31K polypeptide in the fraction obtained after the first sucrose gradient, indicating that this represents part of the active enzyme complex (Fink et al., 1990).

As far as we are aware glucan synthase-II has so far not been purified to homogeneity from any source.

3.0 MATERIALS AND METHODS

3.1 MATERIALS

UDP-[^{14}C] glucose (296 Ci/mol), GDP-[^{14}C] mannose and UDP-[^{14}C] N-acetylglucosamine were purchased from the Radioc- hemical Centre, Amersham (U.K.). Unlabeled UDP-glucose, various nucleotides, adenosine, glucose-6-phosphate, Tris, n-octyl- β-D-glucoside, Triton X-100, sodium dodecyl sulphate (SDS), CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1 propane- sulfonate), Nonidet P-40, cellobiose, bovine serum albumin, DEAE-sephadex, sephadex G-150, DEAE-cellulose, amino acid standards and Freund's adjuvants were purchased from Sigma Chemical Company (St. Louis, USA). Acrylamide, N, N'-methylene-bis-acrylamide and TEMED were obtained from Serva (FRG). Bacterial α-amylase and β-amylase, digitonin, β-mercaptoethanol and ammonium persulphate were procured from E. Merck. Hydroxylapatite gel material for column chromatography and the molecular weight standards were obtained from Biorad (USA). The protein-A insoluble from Staphylococcus aureus was acquired from Fluka, Switzerland. The exo-(1,3)-β-D-glucanase from Sporotricum dimorphosphorum (Basidiomycetes QM 806) and endo-(1-3)-β-glucanase from Rhizopus QM 6789 were a generous gift from Dr. E.T. Rees U.S. Army Laboratories, Natick, Mass., USA. 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 minutes followed

by thorough washing with distilled water to remove the disinfectant. These seeds were then germinated on a moist, 4-layered presterilized 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 water for 12h and then used for further experimentation.

3.2.2 Buffers

Buffer-A — 50 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 3 mM EDTA and 0.01% (v/v) β-mercaptoethanol.

Buffer-B — 25 mM Tris-HCl, pH 7.4 containing 20% (w/w) sucrose and 1 mM β-mercaptoethanol.

Buffer-C — 25 mM Tris-HCl, pH 7.4, containing 1 mM β-mercaptoethanol.

Buffer-D — 50 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ 0.1% digitonin and 1 mM β-mercaptoethanol

Buffer-E — 10 mM sodium phosphate buffer, pH 6.8, containing 1mM β-mercaptoethanol and 0.1% digitonin.

Buffer-F — 10 mM Tris-HCl, pH 7.4, containing 1 mM β-mercaptoethanol and 0.1% digitonin.

3.2.3 Plasma membrane isolation

Unless stated otherwise all operations were carried out at 0-

$4^{o}C$.

Membrane fractions were prepared from germinating cotyledons as described by Sharma et al. (1986). Briefly, peanut cotyledons (200; ca 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 15 min, followed by centrifugation at 135,000 xg for 60 min. The 12,000-135,000 xg microsomal pellet was suspended in 4ml buffer-B, layered carefully over 5 ml of 34% (w/w) sucrose in buffer-C and centrifuged at 80,000 xg for 90 min (Fig. 1). The pellet containing plasma membranes was collected and suspended in 2 ml of buffer-B. Total protein recovered in plasma membrane fraction varied between 30-40 mg/100 gm fresh weight of peanut cotyledons.

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 105,000 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% (w/w) sucrose successively in the same buffer in 25 ml tubes and centrifuged at 105,000 xg for

Fig.1 SEPARATION OF VARIOUS ENDOMEMBRANE FRACTIONS BY DISCONTINUOUS SUCROSE DENSITY GRADIENT CENTRIFUGATION

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

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

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

Step IV Purified plasma membrane after cenrifugation on the above gradient for 120 min at 95,000 x g.

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

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

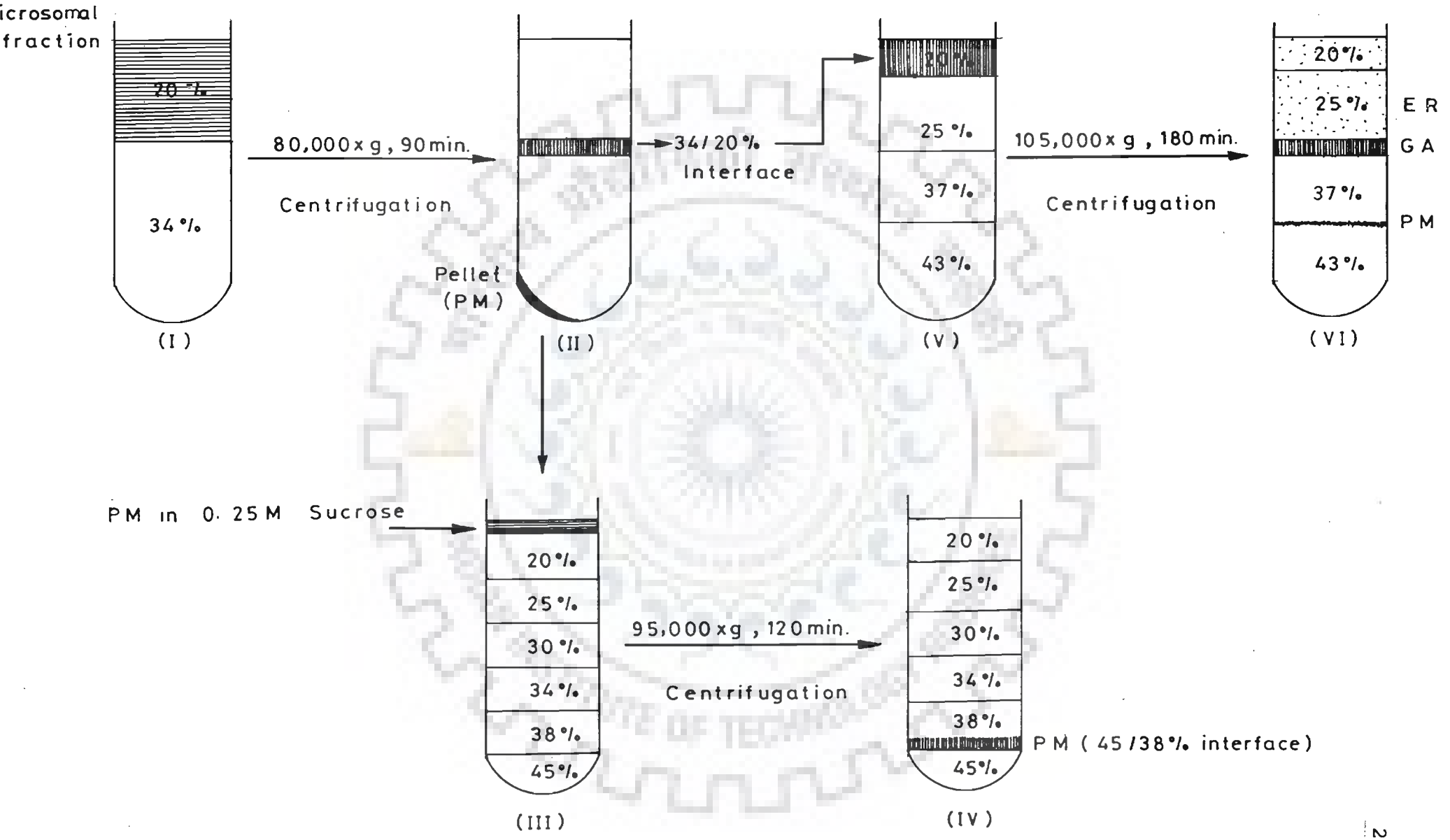
icrosomal
fraction
20 %
34 %
(I)
80,000 x g, 90 min.
Centrifugation
Pellet
(PM)
(II)
34/20 %
Interface
25 %
37 %
43 %
(V)
105,000 x g, 180 min.
Centrifugation
20 %
25 %
ER
GA
37 %
PM
43 %
(VI)
PM in 0.25 M Sucrose
20 %
25 %
30 %
34 %
38 %
45 %
(III)
95,000 x g, 120 min.
Centrifugation
20 %
25 %
30 %
34 %
38 %
PM (45/38 % interface)
45 %
(IV)

Fig. 1

180 min in Centrikon T-2060 centrifuge using the TFT 70.38 Centrikon rotor (Fig. 1). The membrane fraction banding at the interface of 37%/25%, corresponding to a density of 1.15 gm/cm^3, was recovered and diluted to 10% sucrose by buffer-C and pelleted by centrifugation at 105,000 xg for 60 min. The resulting pellet was then suspended in 2-3 ml buffer-B. This fraction represented the enriched GA, free from the cross-contamination of PM and ER (Sharma et al., 1986) as indicated by the distribution of the marker enzymes for PM, GA and ER.

The membrane suspension staying above the 37/25% interface band was diluted to 10% sucrose with buffer-C and centrifuged at 105,000 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 glucan synthase-II (Hall, 1983), Inosine diphosphatase (IDPase) (Green, 1983) and the mannosyltransferase (Lord, 1983) for PM, GA and ER, respectively.

3.2.5 Solubilization of membrane-bound β-glucan synthase

All steps were carried out at 0-4^{o}C. Membrane fraction (100 mg protein) was suspended in 50 ml buffer-B and an equal volume of 1% (w/v) digitonin solution in the same buffer was then added dropwise with gentle stirring followed by three strokes in a teflon homogenizer. The final detergent concentration and the protein-to-detergent ratio were 0.5% and 1:6, respectively. After 30 min incubation in ice the homogenate was centrifuged at 130,000 xg in a Beckman type 50 Ti rotor for 60 min. The clear

supernatant was carefully recovered and the enzyme activity assayed. The pellet was resuspended in buffer-C containing 0.5% (w/v) detergent and assayed. The percent solubilization was calculated assuming the sum of the activities in the supernatant and the resuspended pellet as 100%.

3.2.6 Density gradient centrifugation

Density gradient centrifugation was carried out by the method of Eiberger and Wasserman (1987) with a slight modification. Linear gradients were made by combining 4 ml each of 20% (w/w) and 60% (w/w) sucrose in buffer-D on a gradient maker. Samples of digitonin solubilized fractions of 1.5 ml were layered on the gradient and centrifuged at 200,000 xg for 8 h in Centrikon T-2060 centrifuge using the TST 41.14 Centrikon rotor. After centrifugation the tube was clamped in an up-right position, bottom was pierced with the help of a Kontron piercing unit and fractions (0.6 ml) were collected. An aliquot (0.1 ml) from each fraction was assayed for enzyme activity and protein. Top four peak fractions containing enzyme activity were pooled and subjected to further purification steps.

3.2.7 Hydroxylapatite column chromatography

The hydroxylapatite gel material (20 gm) from Biorad Labs was suspended in 100 ml of 10 mM sodium phosphate buffer, pH 6.8 by a gentle swirling motion and was allowed to swell for 60 min at room temperature. The clear supernatant fluid along with the fine particles of the phosphate gel was decanted off. This process was repeated once. The fully generated gel was packed in a glass column (1.5 x 8 cm) and equilibrated with buffer-E (10

mM phosphate buffer containing 1 mM β-mercaptoethanol and 0.1% digitonin) till the pH of the eluent was 6.8. Routinely, the column was equilibrated overnight at the flow rate of 0.2 $ml.min^{-1}$ in cold ($4^{o}C$). The enzyme fraction (10 ml, 52 mg) obtained from the density gradient centrifugation step was dialyzed overnight against the equlibration buffer (buffer-E) and loaded onto the hydroxylapatite column at a flow rate of 0.1 $ml.min^{-1}$. After washing the column with 20 ml of equilibration buffer, the adsorbed proteins were eluted by a linear gradient from 10 to 500 mM sodium phosphate buffer, pH 6.8, using a single mixing container with 50 ml of buffer-E and a reservoir with 50 ml of 500 mM phosphate buffer containing 0.1% digitonin and 1mM β-mercaptoethanol. Fractions (2.0 ml) were collected at a flow rate of 0.2 $ml.min^{-1}$. Aliquots (0.1 ml) from every other fraction were assayed for protein content and 1,3-β-glucan synthase activity. The peak enzyme-containing fractions were pooled and dialyzed overnight against 200 volumes of prechilled buffer-E and subjected to a second hydroxylapatite column (hydroxylapatite column II) as above, except that 25 ml buffer was used in the mixing as well as in reservoir chambers and 1 ml fractions were collected.

3.2.8 DEAE-sephadex A-50 chromatography

DEAE-sephadex A-50 (10 g) resin was suspended in 200 ml deionized water and allowed to swell overnight in cold (0- $4^{o}C$). The supernatant and the fine particles of the resin were removed. The washed resin was then charged wih 0.5 M NaOH and 0.5 M HCl successively and finally washed with deionized water until the

effluent was neutral. The charged DEAE-sephadex A-50 was suspended in 100 ml of 10 mM Tris-HCl, pH 7.4, containing 0.01% β-mercaptoethanol and 0.1% digitonin (Buffer-F) and packed in a glass column (1x10 cm). The column was equilibrated overnight with the same buffer in cold (0-4^{o}C) at a flow rate of 0.4 ml.min^{-1}.

The dialyzed enzyme (10 ml, 2 mg protein) from the second hydroxylapatite column was applied on the DEAE- sephadex A-50 column (1 x 10 cm) previously equilibrated with buffer-F. The column was washed with 15 ml of buffer-F to remove the unadsorbed proteins.

Adsorbed proteins were then eluted by a linear gradient from 0 to 350 mM NaCl using a single mixing container with 30 ml buffer-F and a reservoir with 30 ml of the same buffer containing 350 mM NaCl. Fractions (1.0 ml) were collected at a flow rate of 0.3 ml.min^{-1}. Aliquots (0.1 ml) from alternate fractions were assayed for protein content and enzyme activity. The peak enzyme-containing fractions were pooled.

3.2.9 Polyacrylamide gel electrophoresis (PAGE)

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

Gel electrophoresis in the presence of SDS was carried out by the procedure of Laemmli (1970) using either gel rods (9 cm length x 0.5 cm diameter) or gel slabs (14 x 14 x 0.2 cm) with eight wells of about 1 cm width with 4 mm spacing in between two consecutive wells. All reagents were prepared in double-

distilled deionised water. Various solutions used were as following :

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

Working solution for preparation of gel

***Separating gel**

	7.5%(w/v)	10%(w/v)
Solution-A	9.00 ml	12.00 ml
Solution-B	9.00 ml	9.00 ml
TEMED	0.02 ml	0.02 ml
Water	18.00 ml	15.00 ml
Solution-D	0.14 ml	0.14 ml

***Stacking gel**

	4.5% (w/v)
Solution-A	1.80 ml
Solution-C	3.00 ml
TEMED	0.02 ml
Water	7.20 ml
Solution-D	0.36 ml

*The working solutions for separating and stacking gels were deaerated prior to 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) followed by heating for 5 min in a boiling water bath. Reference proteins, used for the molecular mass determination were also subjected to the same treatment. After cooling to room temperature 20μl of 0.6% bromophenol blue (tracking dye) was added to each protein sample, including the molecular weight standards. 50 μl protein samples were then layered in the wells of slab gel through the electrophoresis buffer. Electrophoresis was first carried out at a constant current of 10 mA until the sample was concentrated at the interface of the stacking and separating gels. Once the sample was concentrated at the interface, the current was increased to 30 mA per slab gel and electrophoresis was continued until the tracking dye reached close to the base (1 cm from bottom) of the gel. The direction of the current was from the cathode to the anode. After the run, 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 6h at room temperature.

Gel was destained by washing with destaining solution, methanol-acetic acid-water (25:7:68, v/v/v). Protein bands were also visualized by silver staining (Davis et al., 1986). Mobilities of different protein bands were determined relative to bromophenol dye using the following equation

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

For the molecular mass determination of the purified enzyme, the relative mobilities of the standard proteins were plotted against the log of molecular weights and the molecular mass of the purified enzyme was determined.

3.2.9.2 Native-PAGE

The native (gel 7.5% (w/v)) electrophoresis was performed under non-reducing and non-denaturating conditions at a constant current of 30 mA/gel slab at 4°C. Unstained gel was sliced serially into 2 mm thick sections. Each gel slice was minced in an aliquot (500 µl) of enzyme incubation buffer. Gel suspension was agitated at frequent intervals so as to facilitate maximum elution of protein and then centrifuged at 10,000 x g for 10 min. The activity of enzyme was assayed in the eluted gel fraction by adding the substrate. The activity was expressed as radioactivity in cpm incorporated per gel slice.

3.2.9.3 Silver staining

Silver staining of gels was carried out using the method of Davis et al. (1986). Briefly, the procedure is as follows : The gel was fixed in 50% methanol and placed in the orbital shaker for 2h. 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 double-distilled 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 immediately rinsed briefly with water and the 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.

3.2.10 Immunological studies

3.2.10.1 Production of antibodies

Polyclonal monospecific antibodies were made against the purified enzyme in the New Zealand strain white female rabbits following the standard procedures. Rabbits were marked with picric acid for identification as there were two separate forms of enzymes for which immunization was carried out. The animals were bled before immunization from the marginal ear vein to obtain nonimmune (control) serum and to ensure that the animal does not contain high titres of unwanted antibodies. Purified enzyme

sample (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 200 µg protein, was injected subcutaneously into eight sites on the animal's back (0.1 ml/site). The second injection (the booster dose) containing 500 µg protein in Freund's incomplete adjuvant was administered 21 days after the first injection. The injection was prepared with equal volumes (400 µl) of Freund's incomplete adjuvant and the protein antigen and the same eight sites of injection were chosen. The third injection was administered 7 days after the booster dose. In this case 250 µg protein in the Freund's incomplete adjuvant as described above, was used.

3.2.10.2 Collection of antiserum

Five days after the last dose, the rabbits were bled from the marginal ear vein and about 10 ml of blood was collected in 15 ml glass centrifuge tubes. The blood was allowed to clot at room temperature for about 1 h and left overnight at 4°C. This causes the clot to contract. The clot was detached from the walls of the 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 Pasteur pipette and combined with the previous one. The pooled liquid was centrifuged for 20 min at 2,000 xg at 4°C. The clear serum (straw-coloured fluid) was

stored in aliquots at -20°C.

3.2.10.3 Test of antibodies

The antibody formation against the purified enzyme was tested by carrying out the immunoinhibition studies. The antiserum and the preimmune serum were diluted to 1:5, 1:10, 1:20, 1:50, etc., and the ability to inhibit the enzyme activity was investigated using standard enzyme assay procedure with varying concentrations of antiserum. Controls contained an equal amount of the preimmune serum. In classical experiments, enzyme (200 µl) 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 against the enzyme.

3.2.10.4 Partial purification of antibodies

The antibodies (immunoglobulin G, IgG) were purified from the serum of immunized rabbits following the procedure of Mayer and Walker (1987). The serum (5 ml) 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 immunoglobulin-G fraction, was collected with the help of a Pasteur 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 (1 x 8 cm) previously equilibrated in 10 mM phosphate buffer, pH 8.0. The column was washed with equilibration buffer at a flow rate of 0.25 ml/min. Fractions (5 ml) were collected and monitored at 280 nm. IgG was separated in the washings as unadsorbed protein, as an unsymmetrical peak (Fig. 2). 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.

3.2.10.5 Double immunodiffusion

Immunodiffusion was performed on 8 x 4 cm glass slides, coated with 1.5 mm layer of 1% agarose gel, as described by Ouchterlony and Nilsson (1986). 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, which were kept over a flat surface and allowed to solidify at room temperature so that gels of 1.5 mm thickness were formed. A 10 μl well was punched out

Fig.2 PURIFICATION OF IMMUNOGLOBULIN G (IgG)

The partial purification of antibodies was achieved as described in Materials and Methods. IgG was obtained as unadsorbed and unsymmetrical peak monitored by the protein concentration (o—o) at 280 n m. The peak IgG fraction represented fraction numbers 14-16.

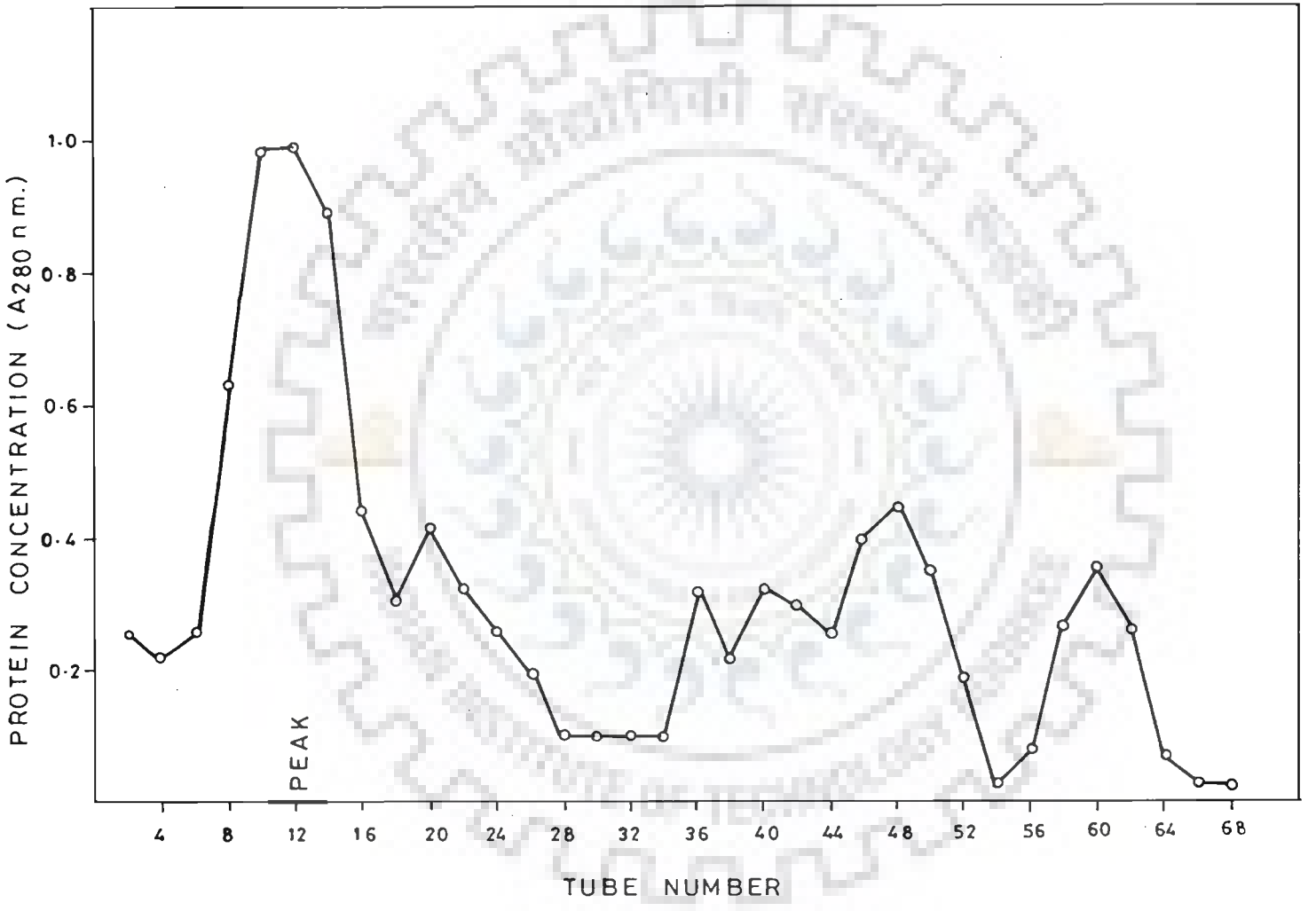
PROTEIN CONCENTRATION (A280 n m.)
1·0
0·8
0·6
0·4
0·2
PEAK
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
TUBE NUMBER

Fig. 2

from the centre of agarose gel and four wells of 10 μl capacity were punched out in a circular fashion around the central well at an equidistance of 1.0 cm from it. The central well was filled with 10μl of the purified 1,3-β-glucan synthase (10 μg protein) and the surrounding wells with antisera of dilution 1:5, 1:10, 1:20, 1:50. In a parallel set, preimmune sera of matching dilutions were used in place of the antisera. The loaded gels were kept overnight (16-18 h) at 37^{o}C in a humid plastic box. The appearance of white precipitin bands between the central well and the surrounding wells indicated the presence of antibodies in antisera. Single precipitin band was indicative of the homogenous antigen (1,3-β-glucan synthase). Precipitin bands did not appear in the control set with preimmune sera. The precipitin bands were stained with 0.5% Coomassie brilliant blue in methanol- acetic acid - H_2O (50:5:45, v/v/v) for 10-15 min and then destained in methanol-acetic acid-H_2O (5:8:87, v/v/v). In some experiments, prior to staining excess protein from the wells was removed by washing with PBS-azide buffer, followed by drying the gel with filter paper sheets at 40^{o}C for 2-6 h.

3.2.11 Determination of H_2 N-terminal amino acid

The determination of the H_2 N-terminal amino acid of the purified enzyme was carried out by the method of Fraenkel-Conrat and Harris (1954). The protein (0.2-0.3 mg) was dissolved in 4 ml of 50% aqueous dioxan. The protein solution was brought to pH 8.7-9.0 with 0.01 N sodium hydroxide and the mixture was stirred for 1.5 h at 40^{o}C with 0.1 ml phenyl isothiocyanate keeping the pH constant. The reaction mixture was then extracted seven times

with benzene and the aqueous solution concentrated to dryness under vacuum over sodium hydroxide.

The sodium salt of the PTC-peptide was redissolved in water (5 ml). Aliquots of 0.25 ml were taken and brought to 0.2-1.0 μM with respect to hydrochloric acid and 0.2-1.0 x 10^{-4} M with respect to peptide by the addition of the correct amounts of water and 5.7N hydrochloric acid. The rate of release of phenylthiohydantoin was determined by following the change of the absorption maximum of the solution from 240 nm or lower, to 265-270 nm during a period of about 2h. The PTH-amino acids were extracted into ethylacetate and the residual peptide was recovered by concentration of the aqueous solution as before. The residue was redissolved in 50% aqueous dioxan and submitted to the same cycle of operations. PTH-amino acids were identified by thin layer chromatography using benzene/ethylacetate (15:3, v/v) as the solvent system. The authentic PTH-amino acid standards were used for comparison of R_F values.

3.2.12 Product characterization

Radioactive products formed during incubation of 1,3-β- glucan synthase under assay conditions were analyzed by testing the susceptibility of radioactive glucan to digestion by specific glucanohydrolases; exo-(1-3)-β-D glucanase (EC 3.2.1.58) and α and β-amylases (EC 3.2.1.1) and (EC 3.2.1.2), respectively as described by Orlean (1982) with slight modification. For 1,3-β-D glucanase digestion radioactive product (35,000 cpm) was incubated with enzyme (0.1 mg/ml) in 0.1 ml and 50 mM sodium

phosphate buffer, pH 6.5 for 18 h at 30°C. Amylase digestion of the radioactive product was carried out in 0.1 ml 100 mM Tris-HCl buffer, pH 7.5, containing 0.1 mg bacterial enzyme/ml. The controls contained radioactive product in 0.1 ml Tris-HCl buffer, pH 7.5, without glucanohydrolases. In other controls amylose and cellulose were used as substrates to check the action of amylases and 1,4-β-glucanase, respectively. After incubation for indicated period, the reactions were stopped by adding 0.02 ml glacial acetic acid.

The whole digest was then chromatographed on Whatman paper No. 1 by descending chromatography using n-butanol:ethylacetate : acetic acid : H_2O (40:30:25:40 v/v) solvent for 37 h. Unlabeled glucose was used as a reference. The radioactive bands on the chromatogram were located by scanning with the help of a TLC linear analyzer, LB 282 (Berthold) fitted with data acquisition system LB 500. The unlabeled sugar bands were detected by alkaline silver nitrate reagent (Trevelyan et al., 1950).
Alternatively glucanase digestion of the radioactive ethanol insoluble glucan was stopped by the addition of 1 ml 70% ethanol. The remaining insoluble glucan was separated by centrifugation, washed four times with 70% ethanol and the radioactivity determined.

3.2.13 Enzyme assays

3.2.13.1 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 a slight modification. The standard

incubation mixture, unless stated otherwise, contained the following in a final volume of 100 µl. Tris- HCl (50 mM, pH 7.4), UDP-[^{14}C] glucose (0.1 µCi, 290 Ci/mol), 0.5 mM unlabeled 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 labeled 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 all the 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. For effector studies, enzyme was assayed with and without fixed concentrations of effector, simultaneously. Under the assay conditions the transfer of radioactive glucose from UDP-[^{14}C] glucose to the glucan polymer was linear upto 20 minutes.

3.2.13.2 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: 3mM inosine diphosphate (IDP); 1 mM $MgCl_2$; 50 mM

Tris-HCl, pH 7.5 and 0.1 ml (100 μg) 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).

3.2.13.3 Assay for mannosyltransferase activity

The mannosyltransferase activity was assayed by the method of Lord (1983). 1ml of membrane fraction was adjusted to 10mM $MgCl_2$ and the reaction was started by adding 0.02-0.2 μCi of GDP-[^{14}C] mannose (Sp. Act. 228 Ci/mol) and incubated 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.

3.2.14 Other methods

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

4.0 RESULTS

4.1 SUBCELLULAR FRACTIONATION OF 1,3-β-D-GLUCAN SYNTHASE ACTIVITY

To localize and purify the 1,3-β-D-glucan synthase (GS-II) activity, peanut cotyledon homogenate was fractionated into various subcellular fractions as described under Materials and Methods and shown in Fig. 1. As shown in Table I, about 72% of the total GS-II activity in the cell-free homogenate (5,000 x g supernate) was found in the post-mitochondrial supernate. When the latter was centrifuged at 135,000 xg for 60 min, approximately 89% of the GS-II activity was fractionated in the microsomal pellet (crude microsomes) largely consisting of plasma membranes (PM), Golgi apparatus (GA) and the endoplasmic reticulum (ER). These results indicated that GS-II was a membrane associated enzyme. In order to determine the specific localization of GS-II, the crude microsomes were fractionated further into PM, GA and the ER by using sucrose gradient centrifugation, and the total as well as the specific activities were determined in individual subcellular fractions. It was found (Table I) that, of the total microsomal GS-II activity, about 78% was associated with the PM and virtually there was no GS-II activity in the GA or the ER fractions. In terms of specific activity, the PM was enriched 10.6-fold compared to the crude microsomes. In addition, the distribution of the marker enzymes (Table I) clearly shows that PM fraction was free from cross-contamination of GA or the ER as the activity of mannosyltransferase (marker enzyme for the ER) and the activity of inosine diphosphatase (marker enzyme for the GA) in PM was very low compared to the GS-II which is also a marker enzyme for

TABLE I

SUBCELLULAR FRACTIONATION OF 1,3-β-D-GLUCAN SYNTHASE (GS-II) ACTIVITY

7-day old germinating cotyledons (ca 80 g fresh weight) were used to prepare various subcellular fractions as described under Materials and Methods. Mannosyltransferase (MTase), Inosine diphosphatase (IDPase), and 5'-Nucleotidase were used as the marker enzymes for the ER, GA and PM fractions, respectively.

Fraction	GS-II activity			Activity of marker enzymes		
	Total activity	Specific activity	% Total activity[a]	MTase	IDPase	5'-Nucleotidase
	cpm x 10^{-3}	cpm/mg/min x 10^{-3}		cpm/mg/min x 10^{-3}	μmoles/mg/min	μmoles/mg/min
Cell-free homogenate (5,000xg supernate)	35714	4.9	100	n.d.[b]	n.d.[b]	n.d.[b]
Post-mitochodrial frac. (5,000-12,000xg supernate)	25714	3.8	71.9	n.d.[b]	n.d.[b]	n.d.[b]
Crude microsomes (12,000-135,000xg pellet)	22857	44.4	64.0 (89.0)	61.28	14.8	26.6
PM	17857	469.9	50.0 (69.5)	3.08	6.5	106.8
GA	665	38.0	1.8 (2.5)	1.87	100.3	37.9
ER	1130	47.5	3.2 (4.4)	533.1	7.9	26.8

[a] Values in parentheses are based assuming that the GS-II activity in post-mitochondrial supernate is 100%

[b] n.d. = not determined

the plant PM (Ray, 1977; Hall, 1983).

4.2 EFFECT OF VARIOUS DETERGENTS ON THE MEMBRANE-BOUND GS-II ACTIVITY

In order to find out a suitable detergent that would solubilize the membrane-bound GS-II activity in stable and active form, effect of five different nonionic detergents, namely n-octyl-β-glucoside, Triton X-100, Nonidet P-40, digitonin and CHAPS on the membrane-bound enzyme was examined. The results are summarized in Table II. It was found that octylglucoside, Triton X-100 and Nonidet P-40 strongly inhibited the GS-II activity and at 0.5% detergent concentration nearly three-fourth enzyme activity was lost. In contrast, however, under identical conditions digitonin and CHAPS stimulated the GS-II activity by 2.4-fold and 1.6-fold, respectively. These results indicated that digitonin or CHAPS would be quite suitable for the solubilization of the PM-bound GS-II activity.

4.3 PURIFICATION OF PM-BOUND 1,3-β-D-GLUCAN SYNTHASE

The purification scheme for the GS-II is shown in Fig. 3 with the data for each fractionation stage in Table III. One of the important steps of the purification scheme is the preparation of a high-specific activity PM fraction. Results shown in Fig. 4 clearly show that the activity of the PM-bound GS-II in peanut cotyledon increased with germination period, reaching a peak level on 7^{th} day after imbibition of seeds. At this physiological state, the total as well as the specific activity of the enzyme increased approximately 3.5-and 2.0-folds,

TABLE II

EFFECT OF VARIOUS DETERGENTS ON THE ACTIVITY OF PLASMA MEMBRANE - BOUND 1,3-β-D-GLUCAN SYNTHASE.

Detergent	Conc.	CMC[a]	1,3-β-glucan synthase activity	Per cent of control
	%	%	nmole.min^{-1}.mg^{-1}	%
None	-	-	106.0	100
β-Octylglucoside	0.5	0.73	26.5	25.0
Triton X-100	0.5	0.02	23.3	21.9
Nonidet P-40	0.5	0.02	25.4	23.9
Digitonin	0.5	0.02	261.6	246.7
CHAPS[b]	0.5	0.60	171.4	161.7

[a] CMC = Critical micelle concentration

[b] CHAPS = (3 - [(3-cholamidopropyl) dimethyl ammonio] - 1-propane sulphonate)

Fig.3 PURIFICATION SCHEME FOR GS-II

The different fractionation steps used in the purification of GS-II are cited here. The scheme shows the steps involved in purification after the separation of the post - mitochondrial supernatant from the 7-day germinating peanut cotyledons.

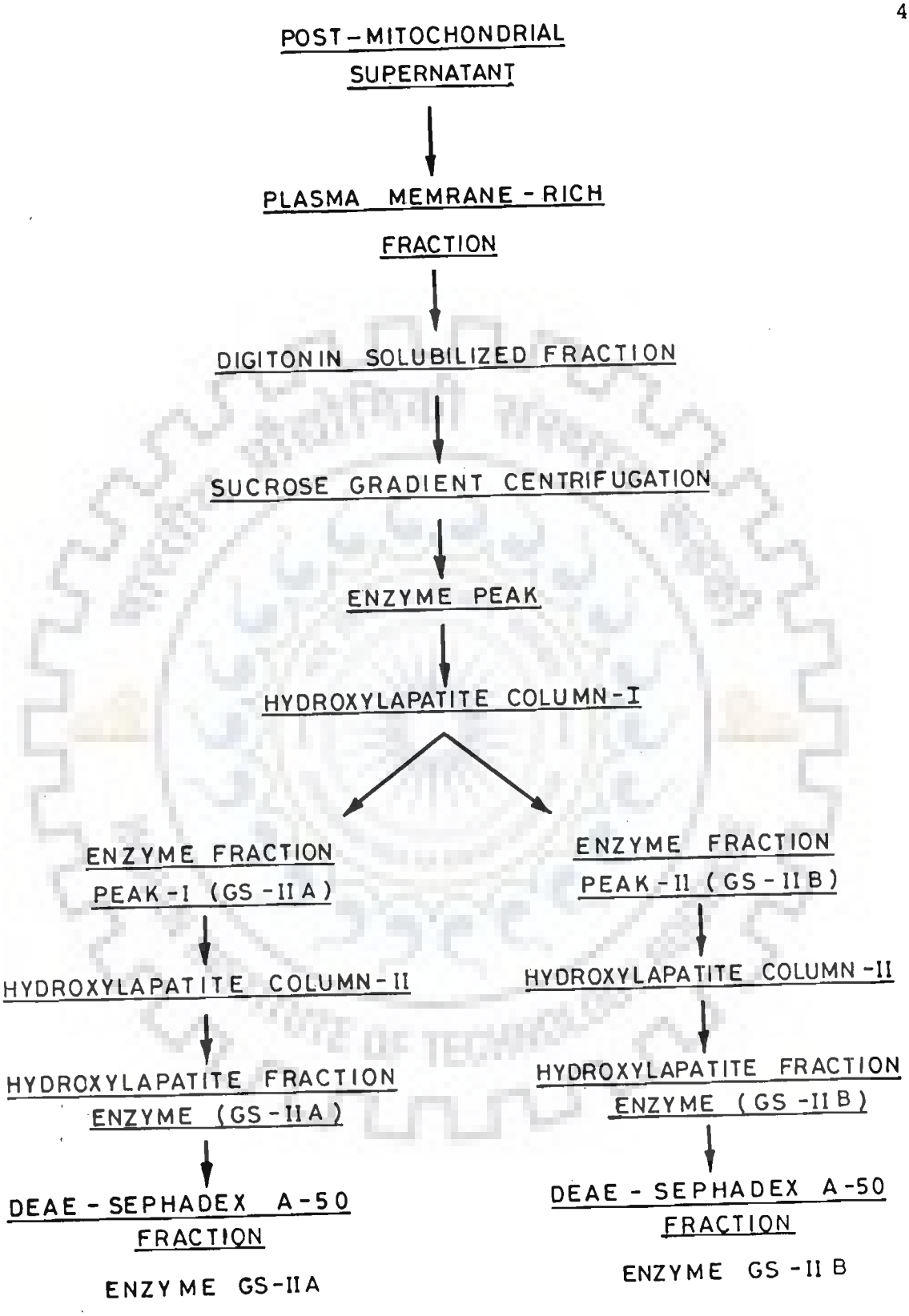
POST-MITOCHONDRIAL SUPERNATANT
PLASMA MEMRANE-RICH FRACTION
DIGITONIN SOLUBILIZED FRACTION
SUCROSE GRADIENT CENTRIFUGATION
ENZYME PEAK
HYDROXYLAPATITE COLUMN-I
ENZYME FRACTION PEAK-I (GS-IIA)
ENZYME FRACTION PEAK-II (GS-IIB)
HYDROXYLAPATITE COLUMN-II
HYDROXYLAPATITE COLUMN-II
HYDROXYLAPATITE FRACTION ENZYME (GS-IIA)
HYDROXYLAPATITE FRACTION ENZYME (GS-IIB)
DEAE-SEPHADEX A-50 FRACTION
DEAE-SEPHADEX A-50 FRACTION
ENZYME GS-IIA
ENZYME GS-IIB

Fig. 3

Fig.4 1,3-β-D- GLUCAN SYNTHASE ACTIVITY AS A FUNCTION OF GERMINATION PERIOD.

Germination was carried out as described in Materials and Methods. The activity of the enzyme 1,3-β-D- glucan synthase (▲——▲) was assayed after different germination period as described under Methods and is represented as nmole x 10^{-1}. min^{-1} /200 cotyledons. Specific activity (●——●) was calculated after assaying the total protein and is represented as nmole.min^{-1}.mg^{-1}.

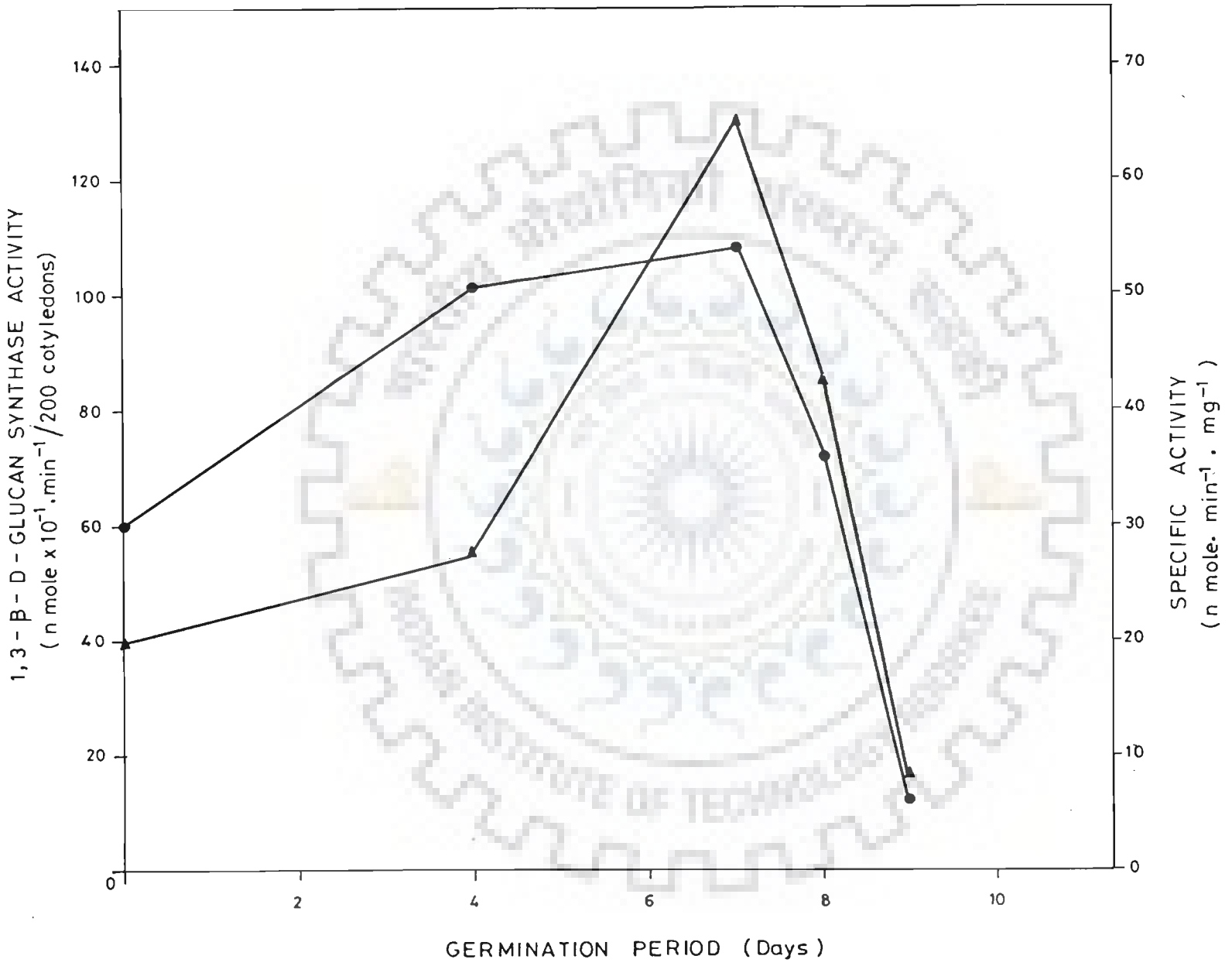
1, 3 - β - D - GLUCAN SYNTHASE ACTIVITY
(n mole x10⁻¹.min⁻¹/200 cotyledons)
SPECIFIC ACTIVITY
(n mole. min⁻¹ . mg⁻¹)
GERMINATION PERIOD (Days)
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60
70
2
4
6
8
10

Fig. 4

respectively. Following this period there was a sharp decline in the level of GS-II activity with only one-fifth of the peak activity remaining on the 9^{th} day of germination, which is several fold lower than the activity level in the dormant seeds. Thus, 7-day old germinating cotyledons were used for the preparation of the high specific activity plasma membrane fraction for the purification of GS-II. The crude microsomal fraction (12,000-135,000 xg pellet) was found to contain high level of GS-II activity with a specific activity of 5.2 units/mg, giving a 10.4-fold purification over the cell-free homogenate (5,000 xg supernate). A further purification of PM was achieved by centrifugation in sucrose step gradients at 95,000 xg for 120 min (Fig. 1). The plasma membrane fraction, separated at 45/38% interface, was found to contain a high specific activity of GS-II (54.7 units/mg) resulting in a 10.5-fold purification over the crude microsomes and an over all purification of 109.4-fold. That this fraction represented mainly plasma membrane, was indicated by the fact that besides glucan synthase-II, which itself is a marker enzyme for the PM, the 5'-nucleotidase, a well-known marker enzyme for the PM (Sharma et al., 1986; Hall, 1983) was also enriched in this fraction with almost no activity of mannosyltransferase and inosine diphosphatase, marker enzymes of the ER and GA, respectively (Table I). These results indicated that the PM fraction was free from GA and ER. Unless stated otherwise, this fraction was therefore used for solubilization and purification of GS-II, in subsequent experiments.

The next step involved the solublization of the PM-bound GS-II with 0.5% digitonin in the presence of 250 mM sucrose and a protein-to-detergent ratio of 1:6. Under these conditions the activity of the solubilized enzyme was significantly higher than the total activity of the PM-bound enzyme before solubilization (Table III). These results clearly indicated the activation of GS-II activity by digitonin. It was not surprizing, however, since even the membrane-bound enzyme activity was stimulated by digitonin (Table II). At this stage the overall yield and purification were 60.9% and 303.6-fold, respectively. Thus, solubilization step has yielded an apparent purification of nearly 3-fold and the preparation of high-specific activity plasma membrane together with solubilization with digitonin gave a 30-fold purification over the crude microsomes and as such these constitute very important steps of the purification scheme. Furthermore, the solubilized enzyme was found to be fairly stable for 15 days at 0-4^{o}C, with about 25% loss in activity (Fig. 5), to allow us further purification. The solubilized enzyme was then subjected to sucrose density gradient fractionation. The activity profile is shown in Fig. 6. It can be seen that, of the enzyme activity applied on the sucrose gradient about two-third activity was concentrated in fraction numbers 6-9, corresponding to 46-34% sucrose gradient. In terms of density the peak fraction (Fr. No. 9) corresponded to 1.1 g/cm^{3}. After this step the purification and yield of GS-II were approximately 620-fold and 39%, respectively.

For further purification the sucrose gradient peak fractions (Fr.

TABLE III

PURIFICATION OF 1,3-β-D-GLUCAN SYNTHASE FROM PEANUT COTYLEDONS.

Results are shown for one preparation of enzyme from 1000g (fresh weight) of 7-day old germinating peanut cotyledons. The enzyme activity was assayed using standard assay procedure as described under Materials and Methods.

		1,3-β-D-glucan synthase			
Fraction	Protein	Total activity	Specific activity	Yield	Purification
	mg.	Units[a]	Units.mg^{-1}	%	- fold
Cell-free homogenate (5,000xg supernate)	82030	41330	0.50	100	1.0
Crude microsomes	5130	26676	5.2	64.5	10.4
Plasma membrane -rich fraction	300	16413	54.7	39.7	109.4
Digitonin-solubilized enzyme	166	25200[b]	151.8	60.9	303.6
Sucrose gradient	52	16112	309.8	39.0	619.6
Hydroxylapatite, I					
Peak I (GS-IIA)	6.7	6405	955.9	15.5	1911.8
Peak II (GS-IIB)	6.5	4251	654	10.3	1308.0
Hydroxylapatite, II					
Peak I (GS-IIA)	1.9	2234	1175.8	5.4	2351.6
Hydroxylapatite, II					
Peak II (GS-IIB)	1.2	1206	1005.0	2.9	2010.0
DEAE-sephadex (GS-IIA)	0.32	1059	3309.4	2.6	6618.8
DEAE-sephadex (GS-IIB)	0.72	240	333.3	0.58	666.6

a. One unit of enzyme activity is the amount required to incorporate one nmole of glucose into the product in one min under standard assay conditions

b. Activation by digitonin is clearly evident.

Fig.5 EFFECT OF STORAGE ON THE SOLUBILIZED ENZYME

The stability of the digitonin solubilized enzyme on storage at $25^{o}C$ and $0-4^{o}C$ was studied. The figure shows the percent residual activity of the digitonin solubilized enzyme stored at $0-4^{o}C$ (■——■) for a period of 15 days and the percent residual activity of enzyme stored at $25^{o}C$ (●——●) for a period of 24 h.

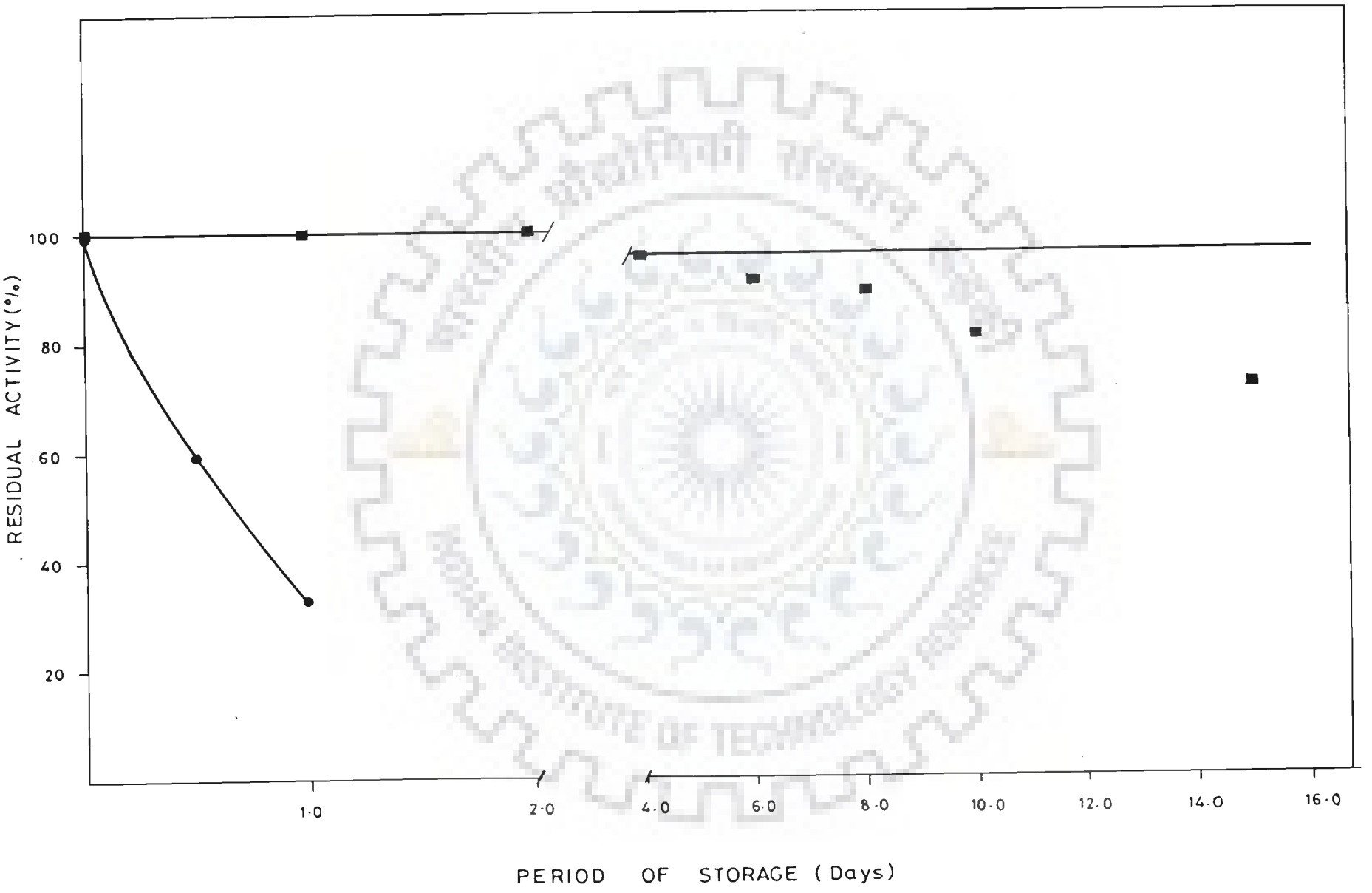
RESIDUAL ACTIVITY (%)
100
80
60
40
20
1·0
2·0
4·0
6·0
8·0
10·0
12·0
14·0
16·0
PERIOD OF STORAGE (Days)

Fig. 5

Fig.6 ACTIVITY PROFILE OF GS-II AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION

The digitonin solubilized fraction was layered onto a linear sucrose gradient of 20% (w/w) to 60% (w/w) sucrose and centrifuged at 200,000 x g for 8 h using the TST 41.14 Centrikon rotor as described in **Materials and Methods**. 1,3-β-D- glucan synthase activity (●——●) and protein (o——o) were determined in the fourteen fractions obtained after centrifugation according to the description in Methods.

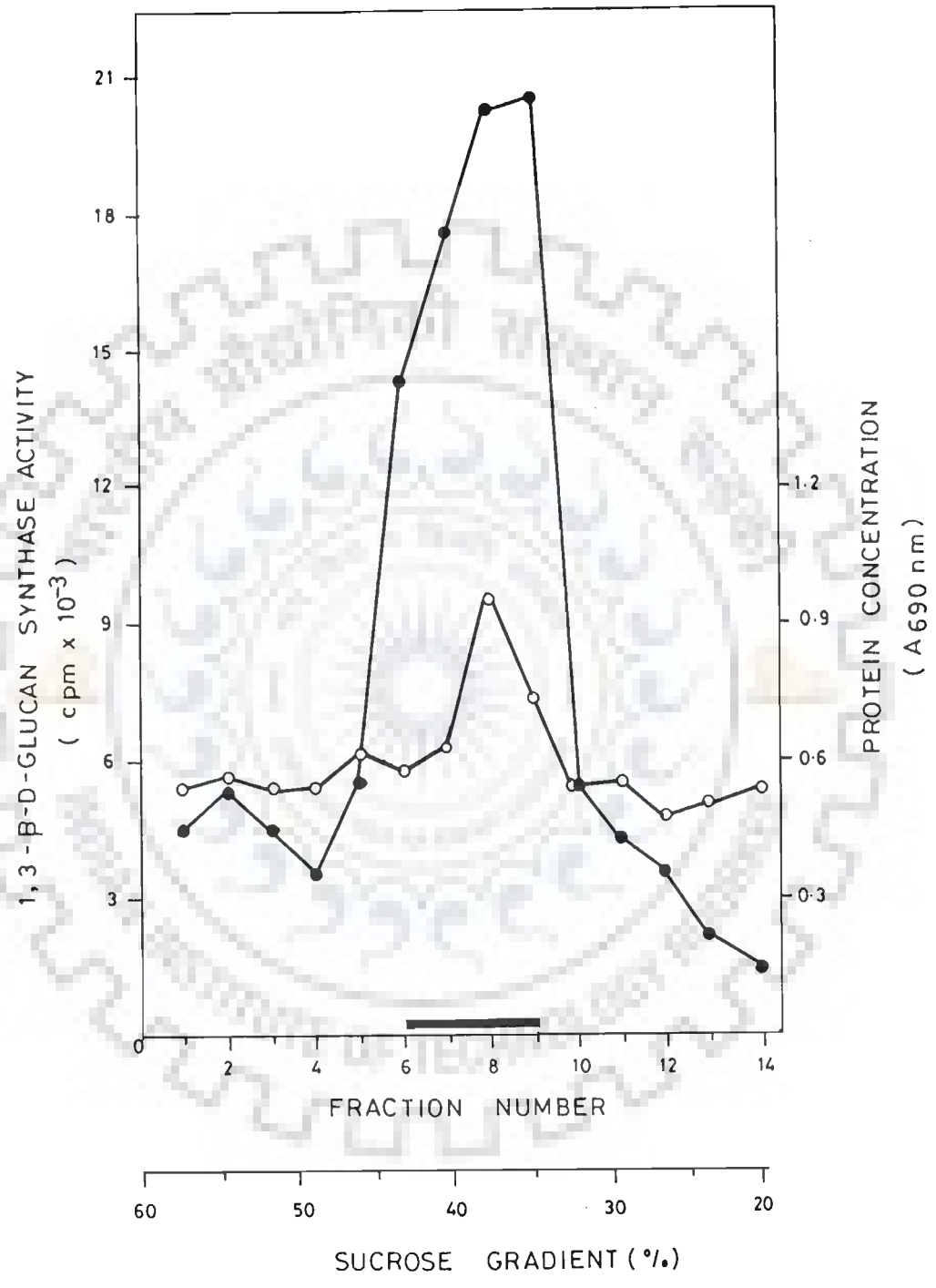
1, 3 - β-D-GLUCAN SYNTHASE ACTIVITY
(cpm x 10^{-3})
PROTEIN CONCENTRATION
(A 690 nm)
FRACTION NUMBER
SUCROSE GRADIENT (%)
0
3
6
9
12
15
18
21
0·3
0·6
0·9
1·2
2
4
6
8
10
12
14
60
50
40
30
20

Fig. 6

No. 6-9) were pooled and dialyzed overnight against 100 volumes of 10 mM phosphate buffer, pH 6.8, containing 0.1% digitonin in cold (0-4^{o}C). The dialyzed fraction was then loaded on a hydroxylapatite column, previously equilibrated with the same buffer. After washing with the equilibrating buffer, the adsorbed proteins were eluted with a linear (10 mM- 500 mM) phosphate gradient as described under Materials and Methods. Fig. 7 shows the elution profile of GS-II activity and proteins. It is very interesting to see that the GS-II activity was eluted in two distinct peaks (peak-I and peak-II), corresponding to 95 mM and 220 mM phosphate concentration. The enzyme activity in these peaks was referred to as GS-IIA and GS- IIB, respectively. The ratio of the activity of GS-IIA to the GS-IIB activity was 60:40, and an overall purification of about 1912-and 1308-fold with an yield of 15.5% and 10.3%, respectively. The GS-IIA and GS-IIB were pooled separately, dialyzed as above and rechromatographed on a second hydroxylapatite column. The results are shown in Fig. 8 and 9. The GS-IIA was eluted at 75 mM phosphate concentration, but the peak was not symmetrical and had a shoulder eluting at approximately 125 mM. At this point it is not clear if this is due to the presence of a third form of GS-II. The major peak fractions (Fr. No. 24-28) were pooled. In this step the purification of GS-IIA increased from 1192-fold to 2351-fold and the yield was reduced from 15.5% to 5.4%.

On rechromatography of GS-IIB on hydroxylapatite column (Fig. 9), the enzyme was eluted in a symmetrical peak at 240 mM phosphate

Fig.7 HYDROXYLAPATITE COLUMN I

The elution profile of the dialyzed sucrose density peak fraction loaded on the hydroxylapatite column. 1,3-β-D-glucan synthase activity (▲——▲) and the protein concentration (●——●) were assayed in alternate fractions. GS-II activity was eluted in two distinct peaks (peak I and peak II), corresponding to 95 mM and 220 mM phosphate concentration.

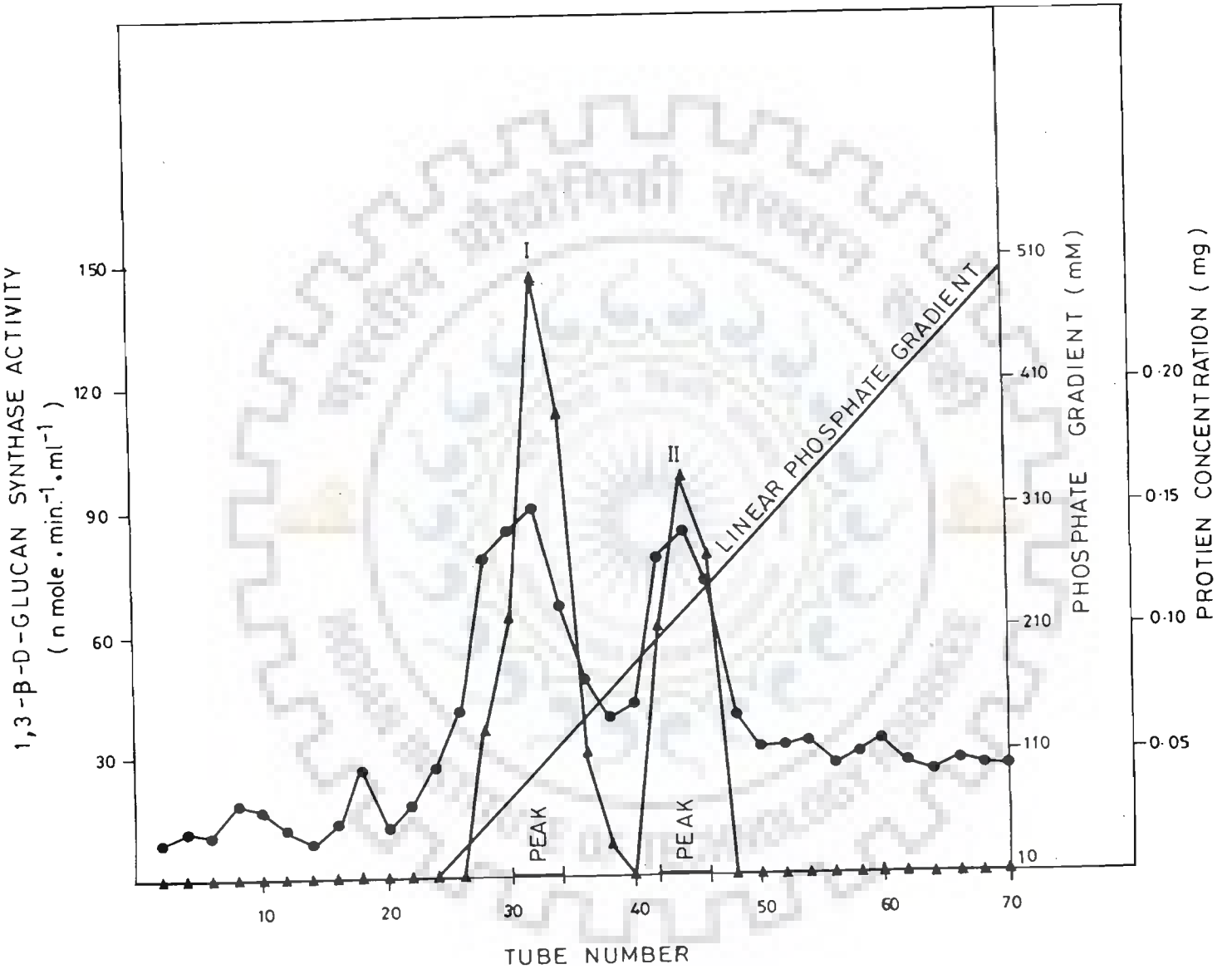
1,3-β-D-GLUCAN SYNTHASE ACTIVITY
(n mole . min.$^{-1}$. ml^{-1})
150
120
90
60
30
I
II
PEAK
PEAK
LINEAR PHOSPHATE GRADIENT
510
410
310
210
110
10
PHOSPHATE GRADIENT (mM)
0·20
0·15
0·10
0·05
PROTIEN CONCENTRATION (mg)
10
20
30
40
50
60
70
TUBE NUMBER

Fig. 7

Fig.8 ELUTION PROFILE OF GS-IIA FROM HYDROXYLAPATITE COLUMN II

Peak I obtained at 95 mM phosphate concentration was dialyzed and rechromatographed on a hydroxylapatite column. The elution profile in the figure shows the GS-IIA enzyme activity obtained at a phosphate concentration of 75 mM and a shoulder eluting at approximately 125 mM. The major peak constituted fractions from 24-28. 1,3-β-D-glucan synthase activity (▲——▲) and the protein concentration (●——●) was determined as described in Materials and Methods.

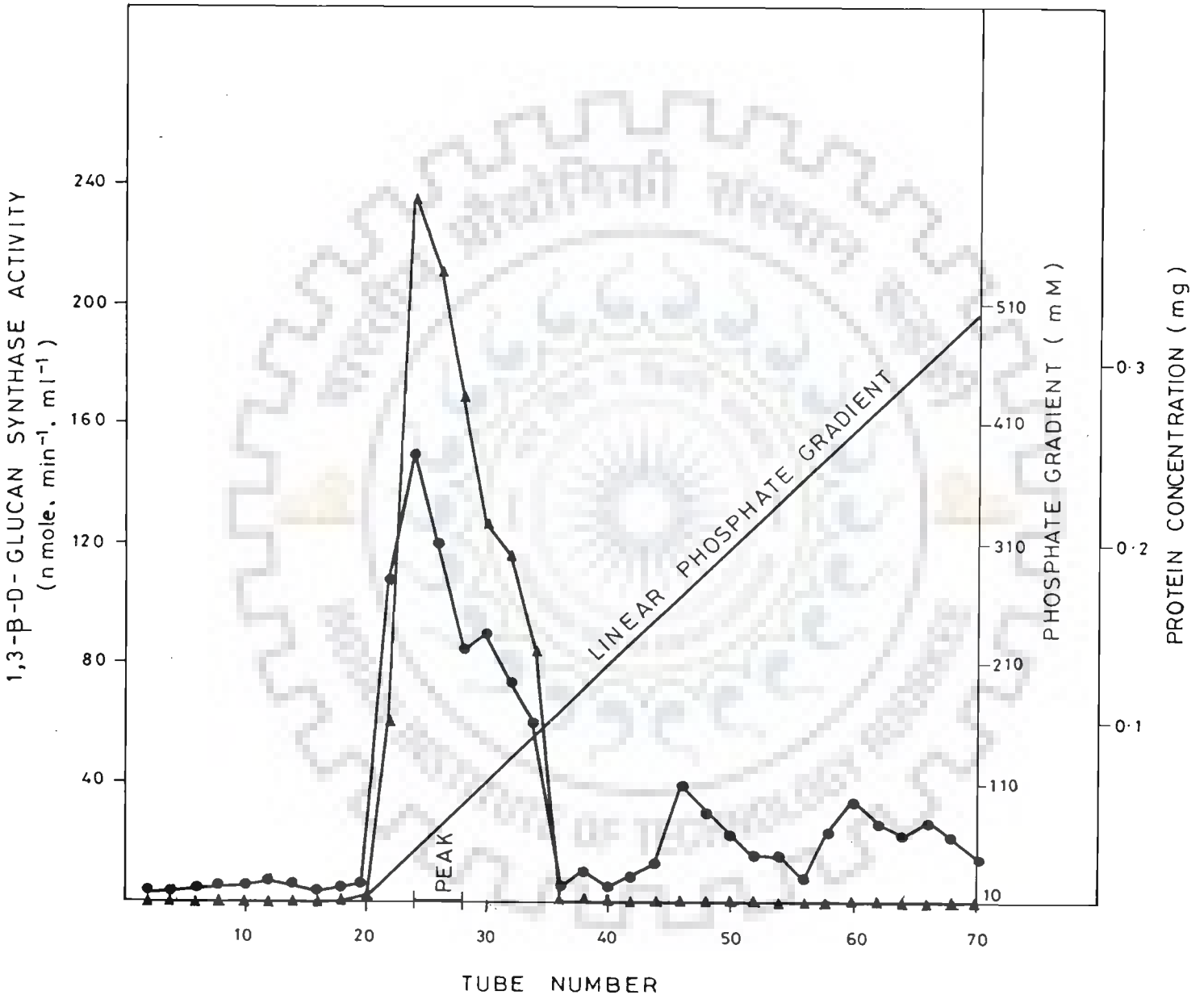
1,3-β-D-GLUCAN SYNTHASE ACTIVITY
(n mole. min⁻¹. ml⁻¹)
240
200
160
120
80
40
PEAK
LINEAR PHOSPHATE GRADIENT
PHOSPHATE GRADIENT (mM)
510
410
310
210
110
10
PROTEIN CONCENTRATION (mg)
0·3
0·2
0·1
10
20
30
40
50
60
70
TUBE NUMBER

Fig. 8

Fig.9 ELUTION PROFILE OF GS-IIB FROM HYDROXYLAPATITE COLUMN II

This is an elution profile of the rechromatography of peak II obtained at 220 mM phosphate concentration from hydroxylapatite column I. The GS-IIB form of the enzyme was eluted in a symmetrical peak at 240 mM phosphate gradient. 1,3-β-D-glucan synthase activity (▲——▲) and the protein concentration (●——●) were determined as described in Materials and Methods.

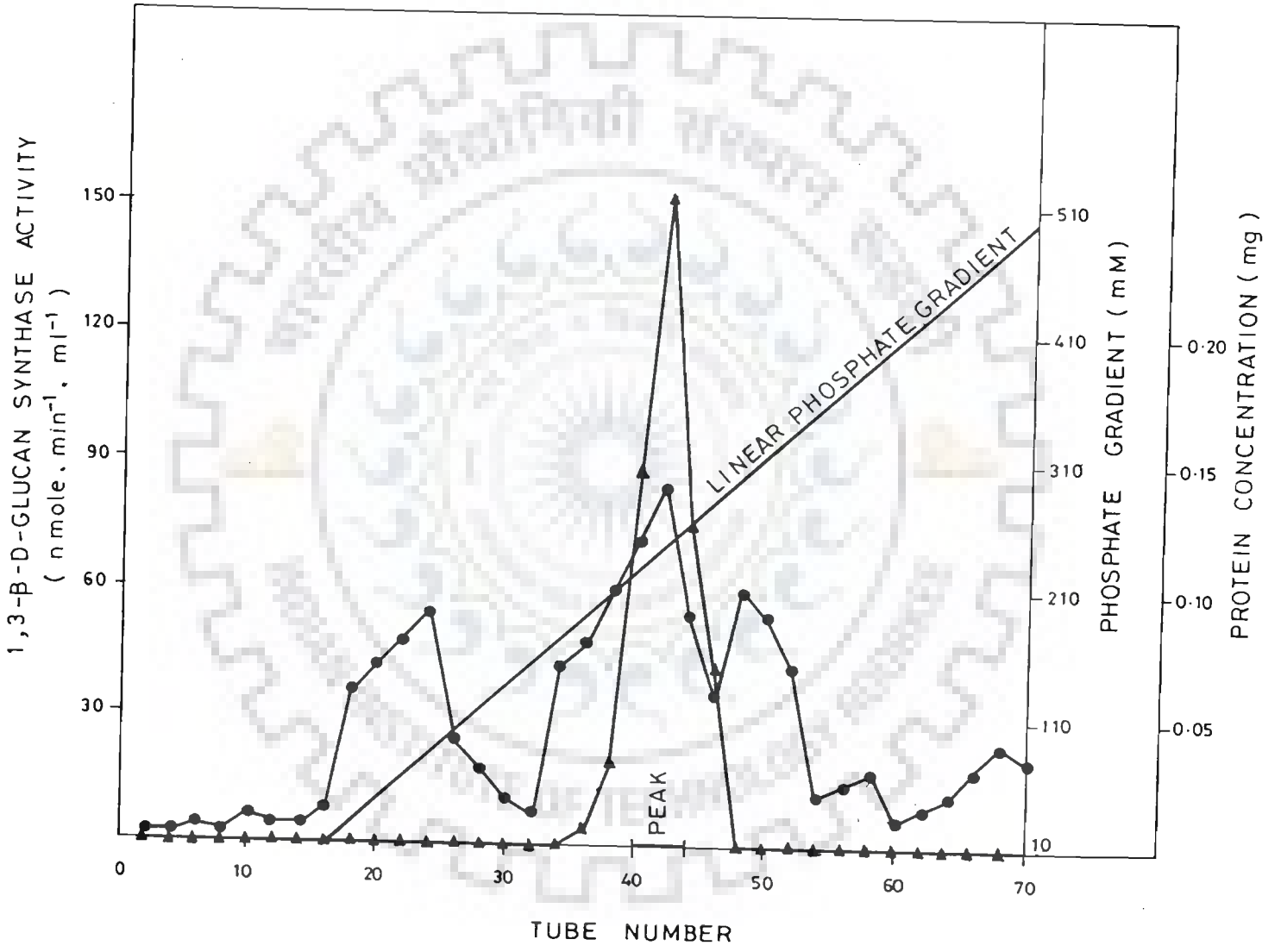
1,3-β-D-GLUCAN SYNTHASE ACTIVITY
(n mole . min⁻¹ . ml⁻¹)
150
120
90
60
30
0
10
20
30
40
50
60
70
TUBE NUMBER
PEAK
LINEAR PHOSPHATE GRADIENT
510
410
310
210
110
10
PHOSPHATE GRADIENT (mM)
0·20
0·15
0·10
0·05
PROTEIN CONCENTRATION (mg)

Fig. 9

concentration (Fr. No. 40-44). These fractions were pooled and the total and specific activity determined (Table III). Following this step, GS-IIB was purified upto 2010-fold with a percent yield of 2.9. Although, on rechromatography on the hydroxylapatite columns the yield of both GS-IIA and GS-IIB was greatly reduced, the purification was siginificant. These steps, therefore, formed the part of the overall purification scheme.

The final purification step involved DEAE-sephadex A-50 column chromatography. Figs. 10 and 11 illustrate the elution patterns of hydroxylapatite purified GS-IIA and GS-IIB, respectively. The GS-IIA activity was eluted in one major peak (Fr. No. 15-17) in the flow-through volume (Fig. 10). However, a sizeable amount of protein was separated in the early flow- through fractions (Fr. No. 7-14). At this stage of purification, the overall purification of GS-IIA was 6618-fold with a percent yield of 2.6.

The GS-IIB was also eluted from the DEAE-sephadex A-50 column in the flow-through volume (Fr. No. 12-17) (Fig. 11). However, unlike GS-IIA, the activity of GS-IIB was greatly reduced with only one-third activity remaining. These results indicated the extreme labile nature of GS-IIB. It seems that some cofactor, like phospholipids, might have been removed during the last step of purification.

4.4 HOMOGENEITY OF GS-IIA AND GS-IIB

4.4.1 SDS-polyacrylamide gel electrophoresis

Fig.10 PURIFICATION OF GS-IIA ON DEAE-SEPHADEX A-50 COLUMN

This is the elution pattern of the hydroxylapatite purified GS-IIA on the DEAE-sephadex A-50 column. The GS-IIA activity was eluted in one major peak (Fr. No. 15-17) in the flow-through volume. 1,3-β-D-glucan synthase activity (▲——▲) and the protein concentration (●——●)were determined as described in Materials and Methods.

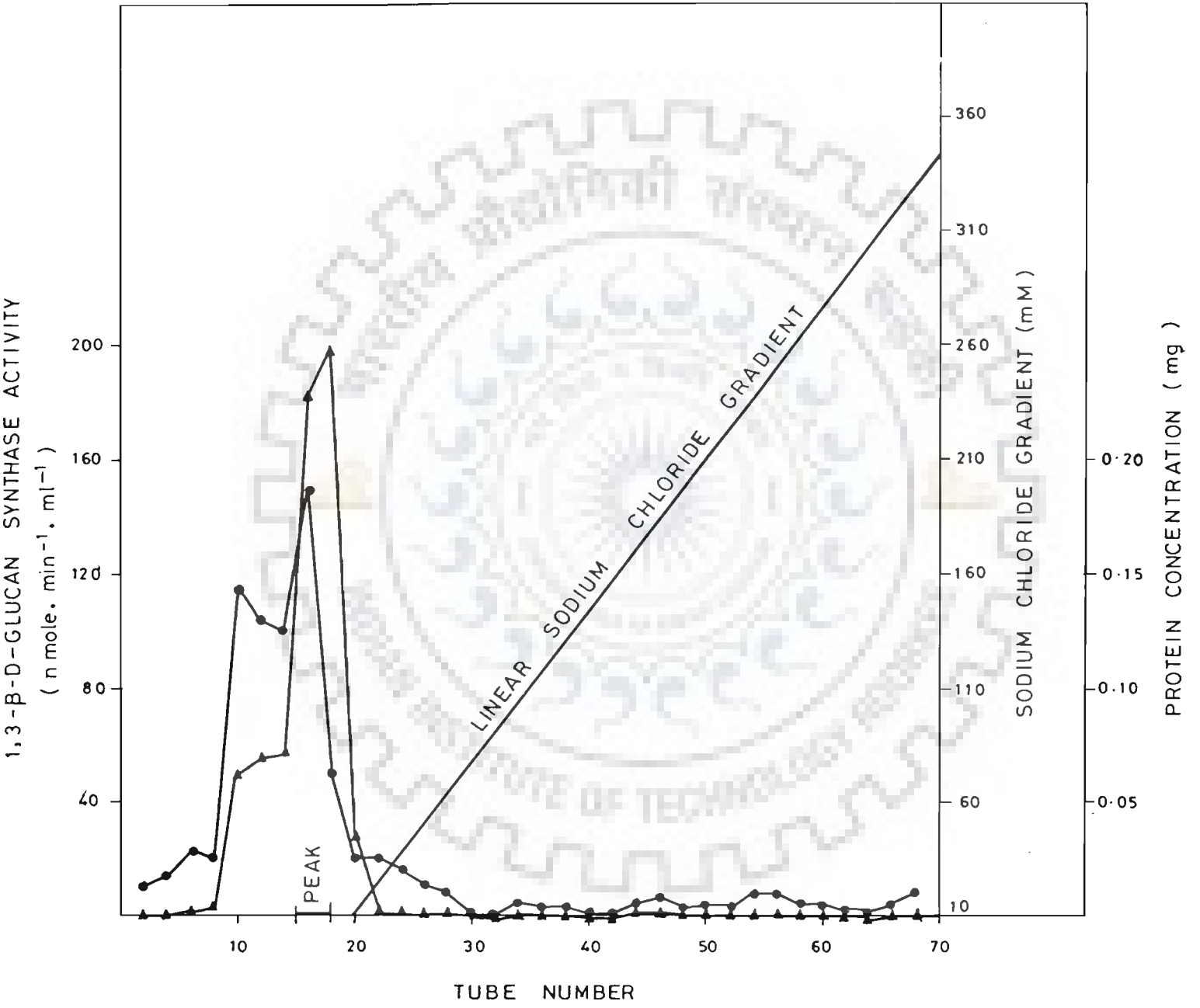
1,3-β-D-GLUCAN SYNTHASE ACTIVITY
(n mole. min⁻¹. ml⁻¹)
200
160
120
80
40
PEAK
LINEAR SODIUM CHLORIDE GRADIENT
360
310
260
210
160
110
60
10
SODIUM CHLORIDE GRADIENT (mM)
0·20
0·15
0·10
0·05
PROTEIN CONCENTRATION (mg)
10
20
30
40
50
60
70
TUBE NUMBER

Fig.11 PURIFICATION OF GS-IIB ON DEAE-SEPHADEX A -50 COLUMN

This is the elution pattern of the hydroxylapatite GS-IIB on the DEAE-sephadex A-50 column. The GS-IIB activity was eluted as a peak (Fr No. 12-17) in the flow-through volume. 1,3 -β-D-glucan synthase activity (▲——▲) and the protein concentration (●——●) was determined as described in Materials and Methods.

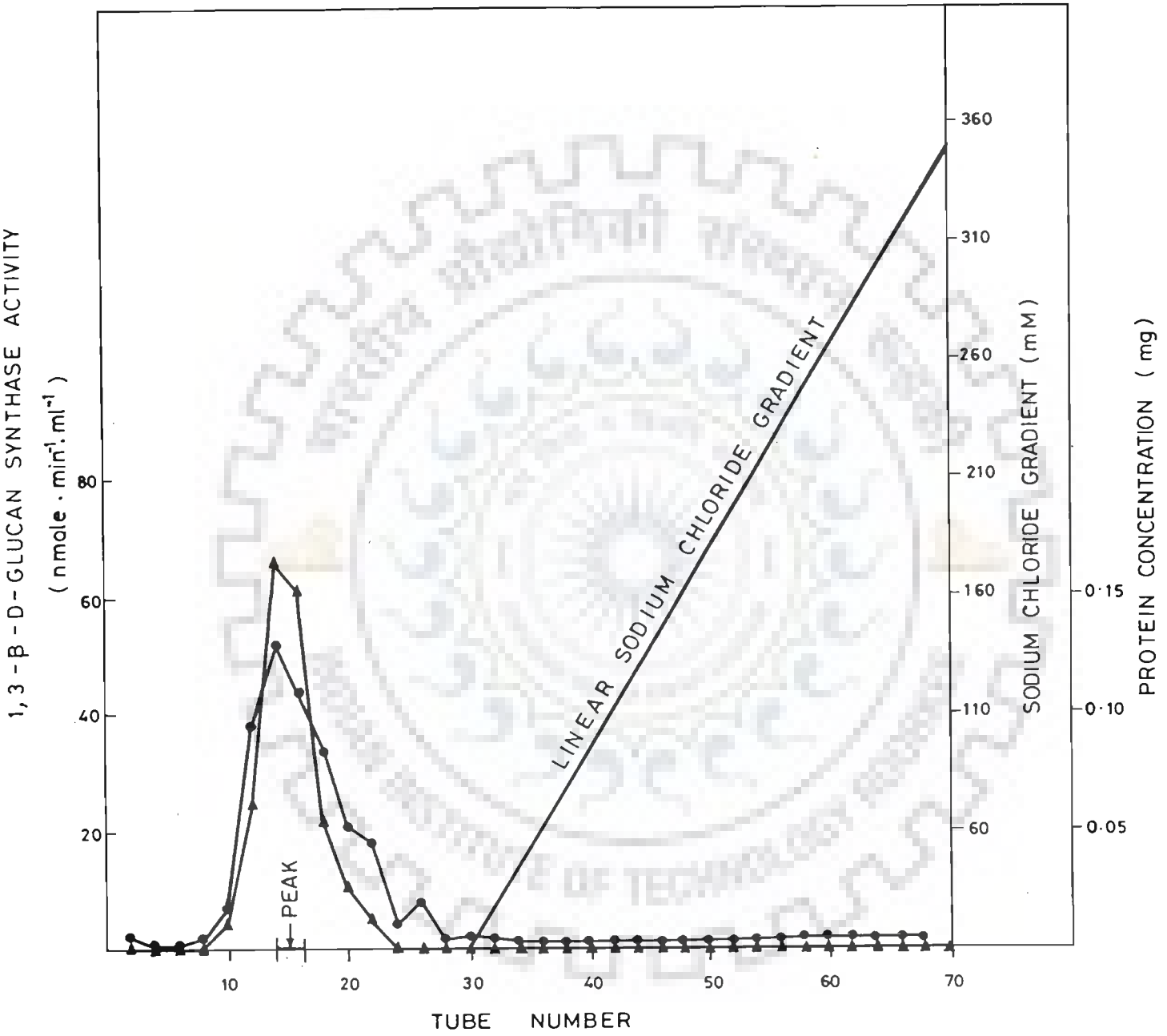
1, 3 - β - D - GLUCAN SYNTHASE ACTIVITY
(nmole . min⁻¹. ml⁻¹)
80
60
40
20
PEAK
10
20
30
40
50
60
70
TUBE NUMBER
LINEAR SODIUM CHLORIDE GRADIENT
360
310
260
210
160
110
60
SODIUM CHLORIDE GRADIENT (mM)
0·15
0·10
0·05
PROTEIN CONCENTRATION (mg)

Fig. 11

The homogeneity of the enzyme fractions obtained at different steps of purification, including the final step, was tested by SDS-PAGE under fully reduced conditions. The results are shown in Fig. 12.A and 12.B. The GS-IIA and GS-IIB fractions eluted from the DEAE-sephadex A-50 column (final step of purification scheme) revealed a single sharp band on the gel, corresponding to apparent molecular masses of about 48K and 57K (Fig. 12.A), respectively. These results indicated that the enzyme preparations were purified to apparent homogeneity. The molecular mass of GS-IIA and GS-IIB were determined by comparison to standard proteins of known molecular weights run simultaneously with the enzyme samples (Fig. 12.A).

4.4.2 Native-polyacrylamide gel electrophoresis

In order to show that the protein band is actually due to GS-II, native-PAGE (without SDS) was performed at 4^{o}C under non-denaturating conditions. The gel was cut into 2 mm slices, individually homogenized in the incubation buffer containing 0.1% digitonin and centrifuged at 10,000 xg for 10 min. The supernatant was assayed for GS-II activity. The native-PAGE showed a single enzyme activity peak which coincided with the single protein band on the gel (Fig. 13). These results confirmed the purity of the enzyme preparation. In the case of GS-IIB enzyme preparation, total activity was lost due to unstable nature of the enzyme (data not shown).

4.4.3 Immunological studies

4.4.3.1 Immunoinhibition

Fig.12.A SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was carried out as described in Materials and Methods to check the homogeneity of the purified GS-IIA and GS-IIB.

Photograph shows the two purified forms along with the molecular weight standards. A represents GS-IIA, B represents GS-IIB. The molecular weight standards are 92.5 K, phosphorylase b; 66.2K, bovine serum albumin; 45K, ovalbumin; 31K, carbonic anhydrase; 21.5 K, soybean trypsin inhibitor; 14.4 K, lysozyme.

Fig. 12.B SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was carried out as described in Materials and Methods with the different fractionation stages of purification along with the molecular weight standards.

The different fractions seen are

A-plasma membrane

B-digitonin solubilized fraction

C-sucrose density gradient fraction

D-purified GS-IIA.

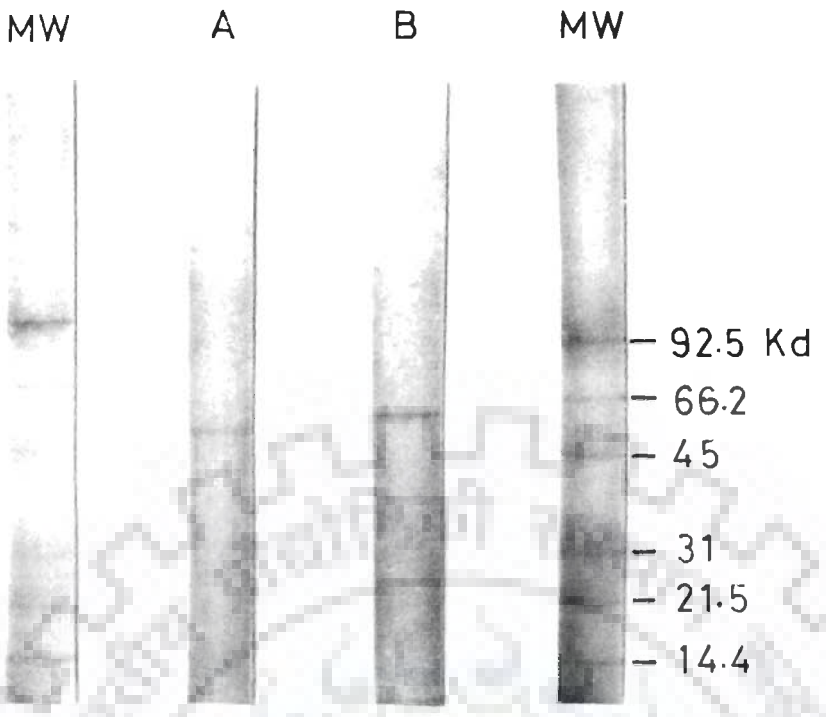

Fig. 12.A

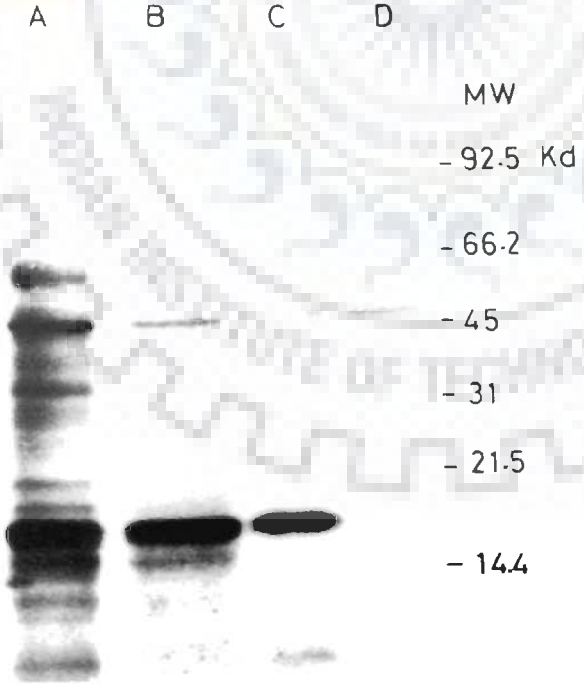

Fig. 12.B

Fig.13 NATIVE-PAGE OF GS-IIA

PAGE was carried out without SDS at 4^{o}C under nondenaturating conditions. 2mm slices were cut, homogenized in 50mM Tris-HCl buffer, pH7.4, containing 0.1 % digitonin and centrifuged at 10,000xg for 10 min. The supernatant was assayed for GS-II as described under Materials and Methods. A,B,C,D,E and F were the standard proteins used. A-phosphorylase b, 92.5K; B-bovine serum albumin, 66.2K; C-ovalbumin, 45K; D- carbonic anhydrase, 31K; E-soybean trypsin inhibitor, 21.5K; F-lysozyme, 14.4K.

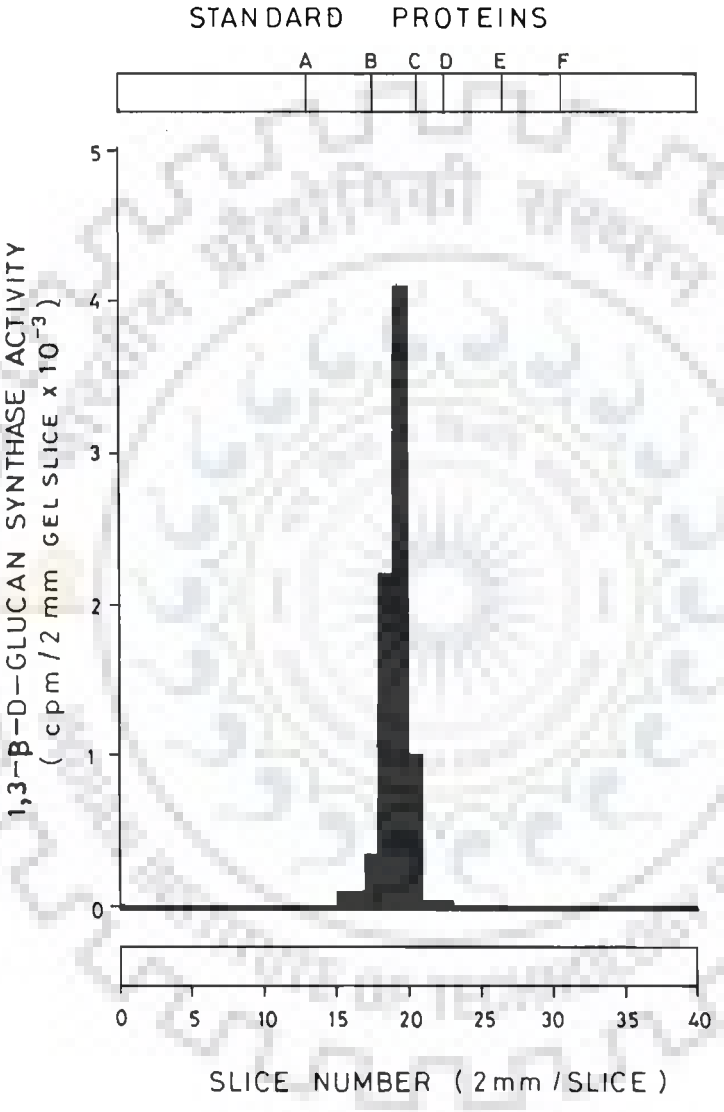
STANDARD PROTEINS
A
B
C
D
E
F
1,3-β-D-GLUCAN SYNTHASE ACTIVITY
(cpm / 2 mm GEL SLICE x 10^{-3})
0
1
2
3
4
5
0
5
10
15
20
25
30
35
40
SLICE NUMBER (2mm / SLICE)

Fig. 13

For immunological studies polyclonal monospecific antibodies were made against the purified GS-IIA and GS-IIB enzyme preparations. The antibody formation was examined by carrying out the immunoinhibition studies. Figs. 14.A and 14.B show the effect of antibody on the activity of GS-IIA and GS-IIB, respectively. The results clearly indicate the formation of antibody against GS-IIA, but not against GS-IIB. Similarly, addition of protein-A sepharose to the enzyme (GS-IIA fraction/antiserum mixture) removed the GS-IIA activity by immunoprecipitation (Fig. 15.A). No inhibition or removal of enzyme activity was observed when nonimmune serum was substituted for the antiserum or when GS-IIB fraction was used instead of GS-IIA fraction (Fig. 15.B). These results clearly show that GS-IIA and GS-IIB are immunologically different and the antibody prepared against GS-IIA was highly specific for the antigen.

4.4.3.2 Immunodiffusion

Although the activity of GS-IIA was specifically inhibited by the polyclonal monospecific antibody, the homogeneity of the antigen could not be confirmed. For this double immunodiffusion experiments were carried out according to the method of Ouchterlony and Nilsson (1986). The single sharp white precipitin band was clearly visible between central well containing the antigen (GS-IIA) and the surrounding wells containing the antiserum of different dilutions. These precipitin bands were visualized by staining with 0.5% Coomassie brilliant blue (Fig. 16.A). As can be seen the intensity of the bands fully matched with the dilution, i.e., the highest intensity band was obtained

Fig.14.A IMMUNOINHIBITION OF GS-IIA AND GS-IIB BY ANTISERUM A

The figure shows the extent of inhibition of GS-IIA and GS-IIB by antiserum A. The preimmune serum A(▲——▲) did not inhibit GS-IIA. The percent residual activity of GS-IIA(●——●) decreased with increasing amount of antiserum A. The percent residual activity of GS-IIB(○——○)showed no change when treated with antiserum A.

Fig. 14.B IMMUNOINHIBITION OF GS-IIA AND GS-IIB BY ANTISERUM B

The figure shows the extent of inhibition of GS-IIA and GS-IIB by antiserum B. The preimmune serum B(▲——▲) did not inhibit GS-IIB. The percent residual activity of GS-IIA (●——●)and GS-IIB (○——○)showed no change when treated with antiserum B.

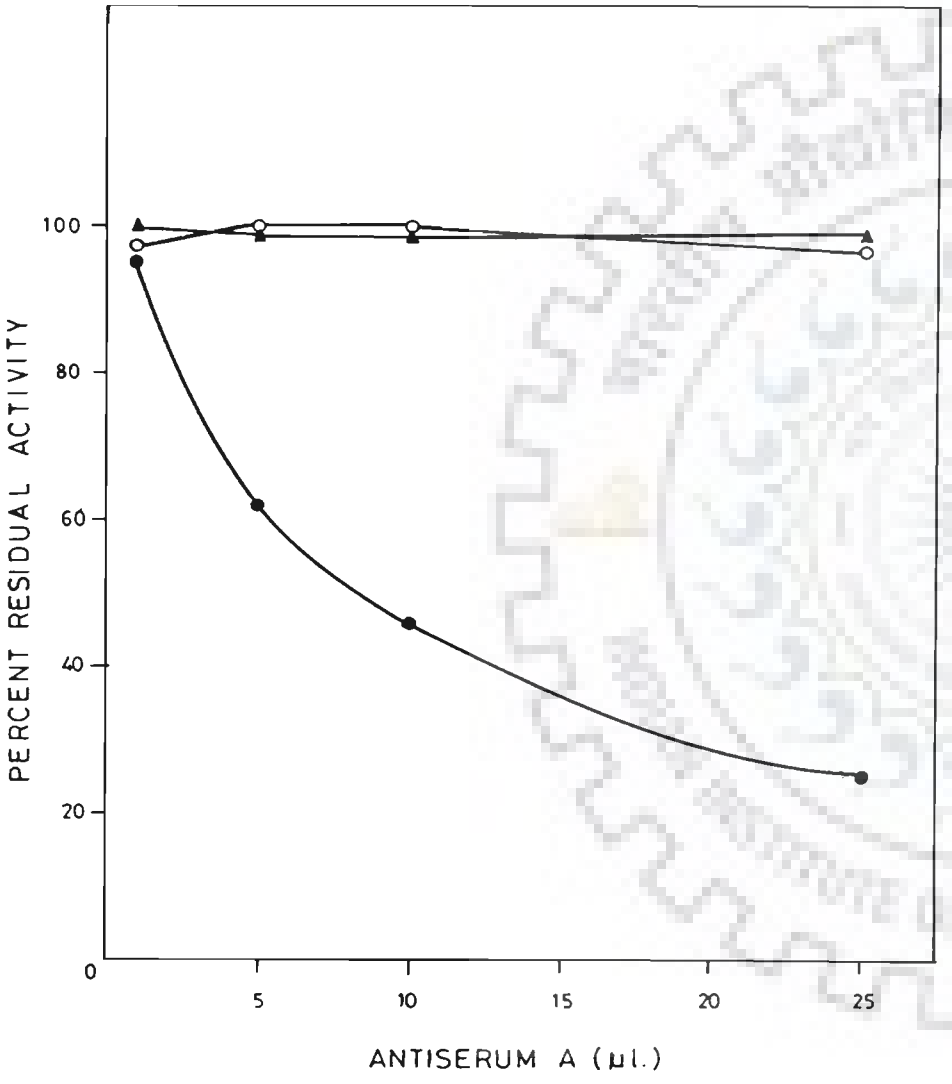

Fig. 14.A

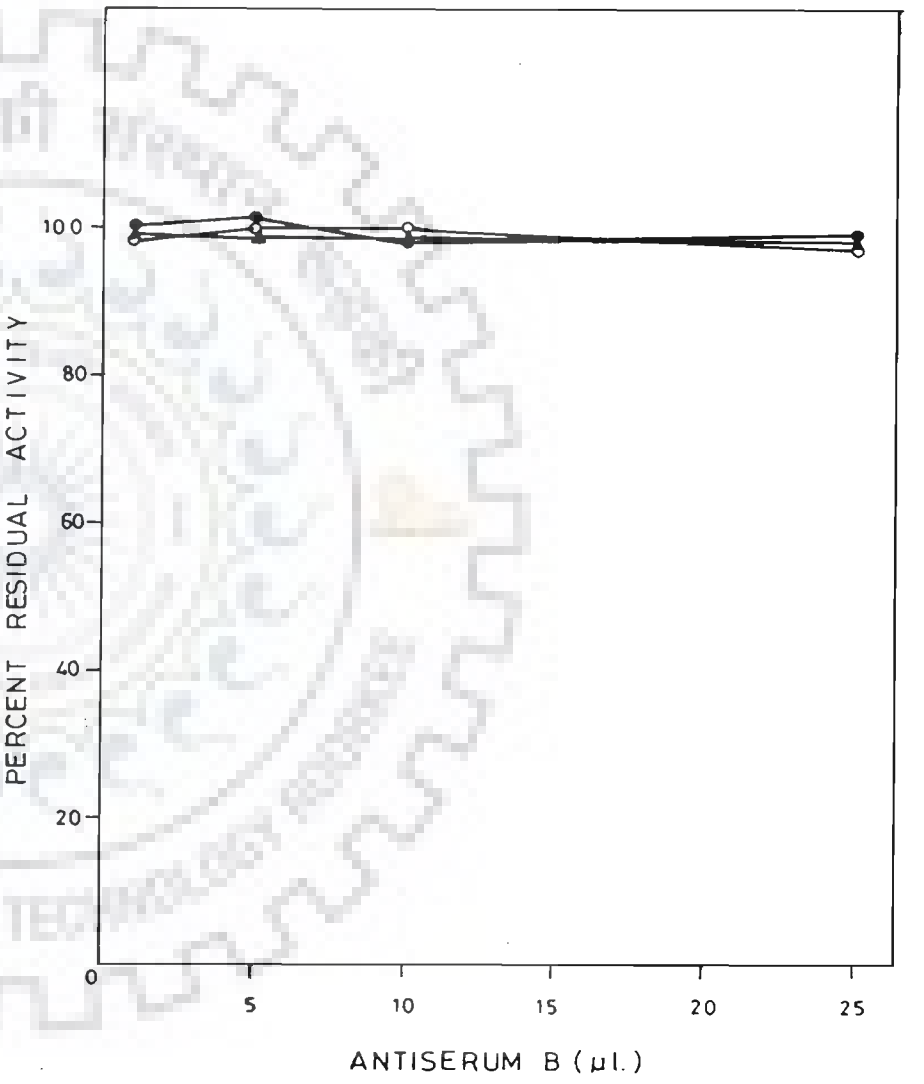

Fig. 14.B

Fig.15.A IMMUNOPRECIPITATION OF GS-IIA WITH PROTEIN-A SEPHAROSE

The figure shows the extent of precipitation of GS-IIA brought about by protein-A/antiserum A complex.

The preimmune serum A + protein - A complex (▲——▲) did not inhibit GS-IIA. The percent residual activity of GS-IIA(●——●) decreased with increasing amount of antiserum A + protein - A complex. The percent residual activity of GS-IIB(o——o) showed no change when treated with antiserum A + protein - A complex.

Fig.15.B IMMUNOPRECIPITATION OF GS-IIB WITH PROTEIN-A SEPHAROSE

The figure shows the extent of precipitation of GS-IIB brought about by protein -A/antiserum B complex.

The preimmune serum B + protein - A complex (▲——▲) did not inhibit GS-IIB. The percent residual activity of GS-IIA (●——●) and GS-IIB (o——o) showed no change when treated with antiserum B + protein - A complex.

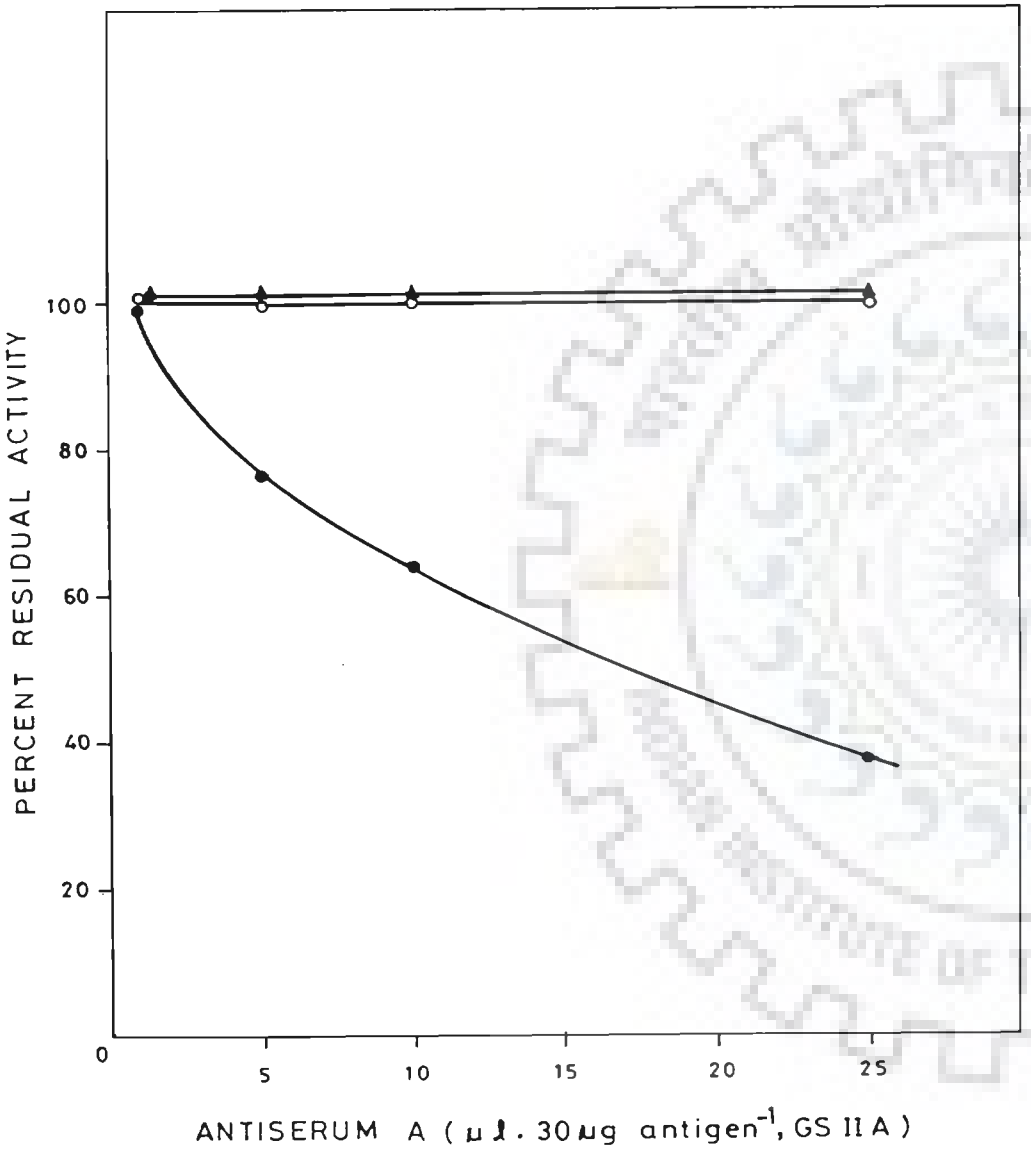

Fig. 15.A

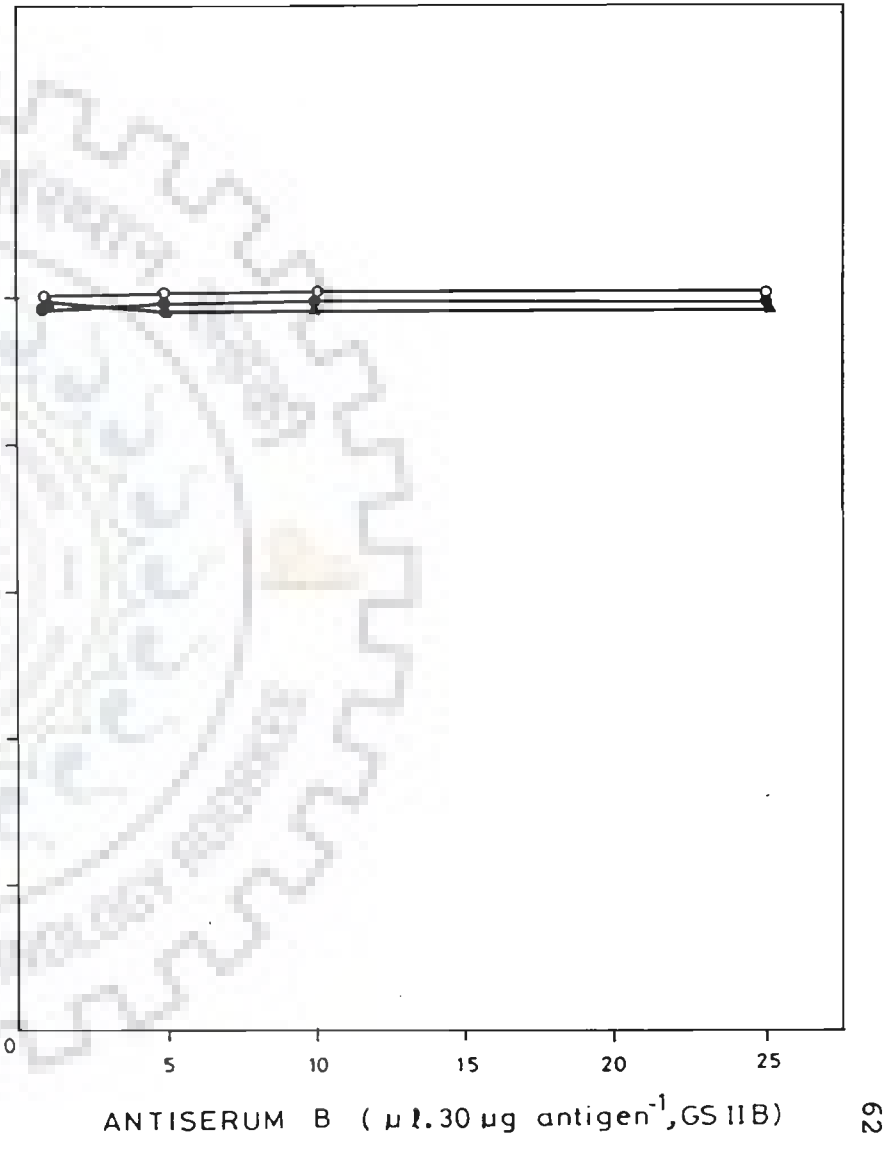

Fig. 15.B

Fig.16.A IMMUNODIFFUSION PATTERNS OF ANTISERUM GS-IIA

Conditions employed for immunodiffusion were those as described under Materials and Methods. The precipitin bands were visualized by staining with 0.5 % Coomassie brialliant blue. As seen in the photograph the different wells contained;

Central well. purified enzyme GS-IIA

Well 1. antiserum A in a 1:5 dilution with PBS-azide.

Well 2. antiserum A in a 1 : 10 dilution with PBS-azide.

Well 3. antiserum A in a 1:50 dilution with PBS-azide.

Well 4. antiserum A in a 1:20 dilution with PBS-azide.

Fig.16.B IMMUNODIFFUSION PATTERNS OF PREIMMUNE SERUM GS-IIA.

Conditions employed were the same as above except that preimmune serum obtained before the immunization schedule was used as a confirmation of antibody production.

As seen in the photograph the different wells contained;

Central well. purified enzyme GS-IIA

Well 1. preimmune serum A in a 1:5 dilution with PBS-azide.

Well 2. preimmune serum A in a 1:10 dilution with PBS-azide.

Well 3. preimmune serum A in a 1:50 dilution with PBS-azide.

Well 4. preimmune serum A in a 1:10 dilution with PBS -azide.

Fig. 16

with antiserum of the lowest dilution. No precipitin band was observed when preimmune serum was substituted for the antiserum (Fig. 16.B). These results clearly showed the homogeneity of the antigen, since no other protein band was observed on the gel.

4.4.4 H_2N-terminal amino acid.

The H_2N-terminal amino acid of GS-IIA and GS-IIB fractions were determined using the Fraenkel-Conrat and Harris method (1954). The phenylthiohydantoin (PTH) derivative of H_2N- terminal amino acid was identified by thin layer chromatography on silica gel plates by comparing the R_F values of standard PTH-amino acid derivatives.

As judged by the R_F value of PTH-derivatives of amino acids (Table IV), leucine and lysine were found to be the H_2N-terminal amino acid in GS-IIA and GS-IIB. The fact that only one PTH-amino acid derivative was found in both GS-IIA and GS-IIB enzyme preparations, indicate that the two enzymes (GS-IIA and GS-IIB) are apparently homogenous, otherwise, in the event of the presence of other proteins more tham one PTH-derivative of amino acids would have been produced. In addition, these results indicate that GS-IIA and GS-IIB are different enzymes and each contains only one polypeptide. However, the presence of more than one polypeptide with same H_2N-terminal amino acid cannot be entirely ruled out in moment of time.

In summary, the results of SDS-PAGE, native-PAGE, double immunodiffusion and H_2N-terminal amino acid determination show that the GS-IIA and GS-IIB have been purified to apparent homogeneity.

TABLE IV

ANALYSIS OF PTH - DERIVATIVES OF H_2N - TERMINAL AMINO ACID OF GS-IIA AND GS-IIB BY TLC

PTH - derivatives were prepared by the method of Fraenkel- Conrat and Harris (1954) which were subsequently separated on TLC as described in **Materials and Methods.** The solvent system used was benzene : ethylacetate (15:3,v/v). The spots were detected and the R_F values were determined by comparison with the R_F values of the standard PTH -derivatives of amino acids.

PTH - derivatives of amino acids.	**R_F value[a]**
H_2N - terminal amino acid of GS-IIA	0.85
H_2N - terminal amino acid of GS-IIB	0.13
L - Gly	0.37
L - Try	0.53
L - Val	0.72
L - Ala	0.63
L - Glu	0
L - Leu	0.85
L - Met	0.58
L - Ileu	0.77
L - Pro	0.79
L - Thr	n.i[b]
L - Tyr	0.33
L - Asp	0
L - Phe	0.58
L - Ser	0.22
L - Lys	0.13

[a] R_F = Distance travelled by a PTH-derivative of amino acid fro origin to the mid-point of the spot./Distance of th solvent front from the origin.

[b] n.i = not identified on the TLC plate

4.5 PRODUCT CHARACTERIZATION

The radioactive products formed during incubation of GS-IIA and GS-IIB under the standard assay conditions were analyzed by testing the susceptibility of the radioactive glucan to digestion by specific glucanohydrolases; exo-(1,3)-β-D-glucanase (EC 3.2.1.58), α-amylase (EC 3.2.1.1) and β-amylase (EC 3.2.1.2). The enzyme-digested radioactive material was tested by descending paper chromatography. It was found that the radioactive product was resistant to α-and β-amylases as the whole radioactivity was completely immobile on paper chromatography and no peak corresponding to glucose, maltose and lower oligosaccharides was detected (Fig. 17.A, B), indicating the absence of glycogen. However, after incubation with the exo-(1,3)-β-D-glucanase for 18-24h at room temperature almost total radioactivity travelled on paper chromatogram with glucose peak (Fig. 17.A, B). The radioactivity product was also resistant to the periodate oxidation, since after periodate treatment, reduction with sodium borohydride, acid hydrolysis and paper chromatography, the entire radioactivity migrated with glucose peak (data not shown). These results clearly indicated that the reaction product formed by GS-IIA or GS-IIB was predominantly β-(1,3)-linked glucan, and if any other linkages were formed, these were undetectable.

4.6 PROPERTIES OF GS-IIA

4.6.1 Molecular size

The molecular mass of GS-IIA as determined by SDS-PAGE under

Fig.17.A PRODUCT CHARACTERIZATION USING HYDROLYTIC ENZYMES

Product characterization was carried out as described in Materials and Methods. The figure shows the radioactivity in cpm vs the distance travelled on chromatogram. The radioactivity that remained at the origin (▲——▲) was the radioactive product resistant to α and β - amylases. The radioactivity that travelled with the glucose peak (●——●) was the product treated with β-(1,3) - glucanase.

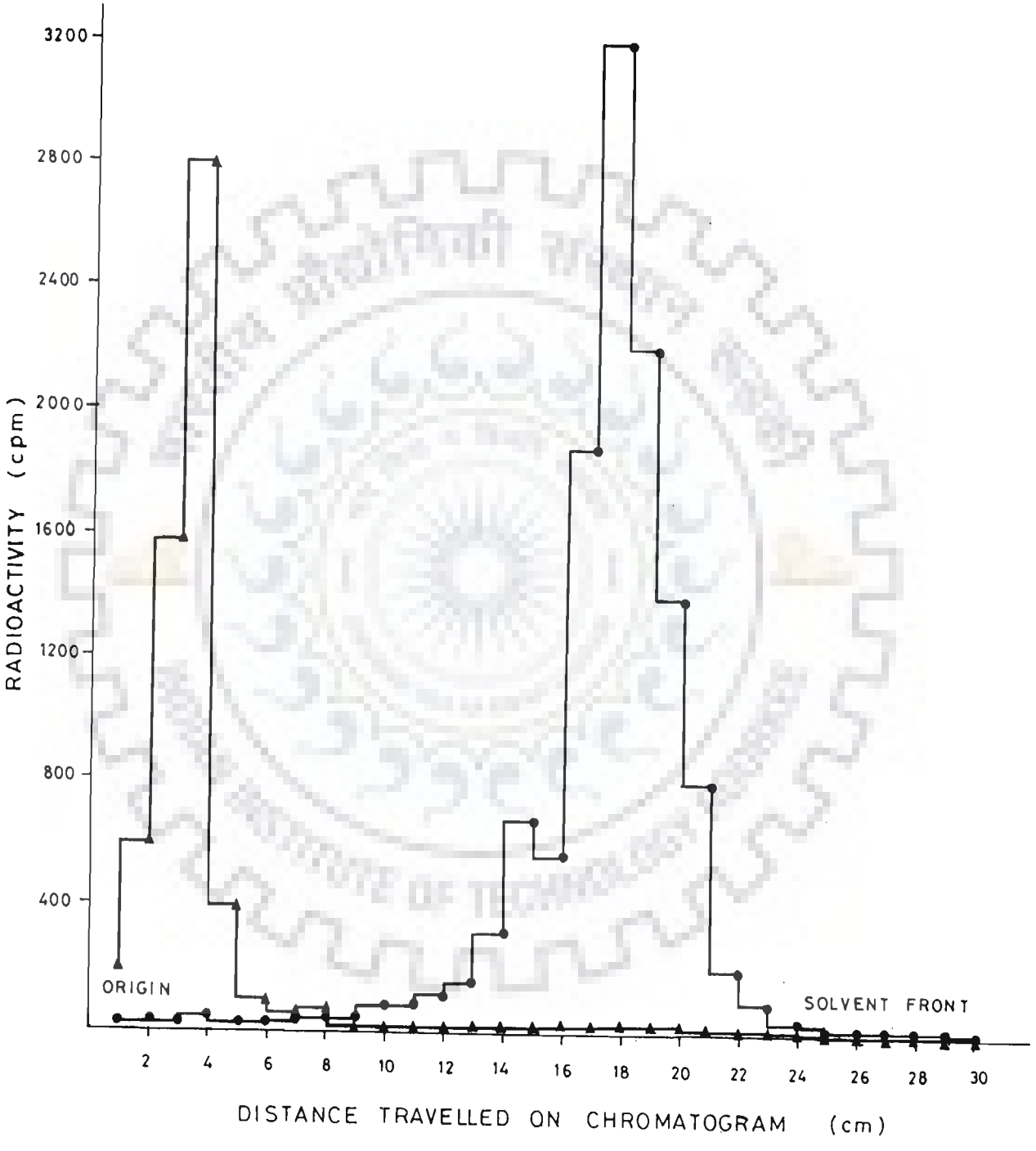
3200
2800
2400
2000
1600
1200
800
400
RADIOACTIVITY (cpm)
ORIGIN
SOLVENT FRONT
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
DISTANCE TRAVELLED ON CHROMATOGRAM (cm)

Fig. 17A.

Fig.17.B PRODUCT ANALYSIS

The top panel shows the TLC linear analyzer scan of the product resistant to α and β-amylases.

The bottom panel shows the TLC linear analyzer scan of the product treated with β-(1,3)-glucanase that travelled with the glucose peak.

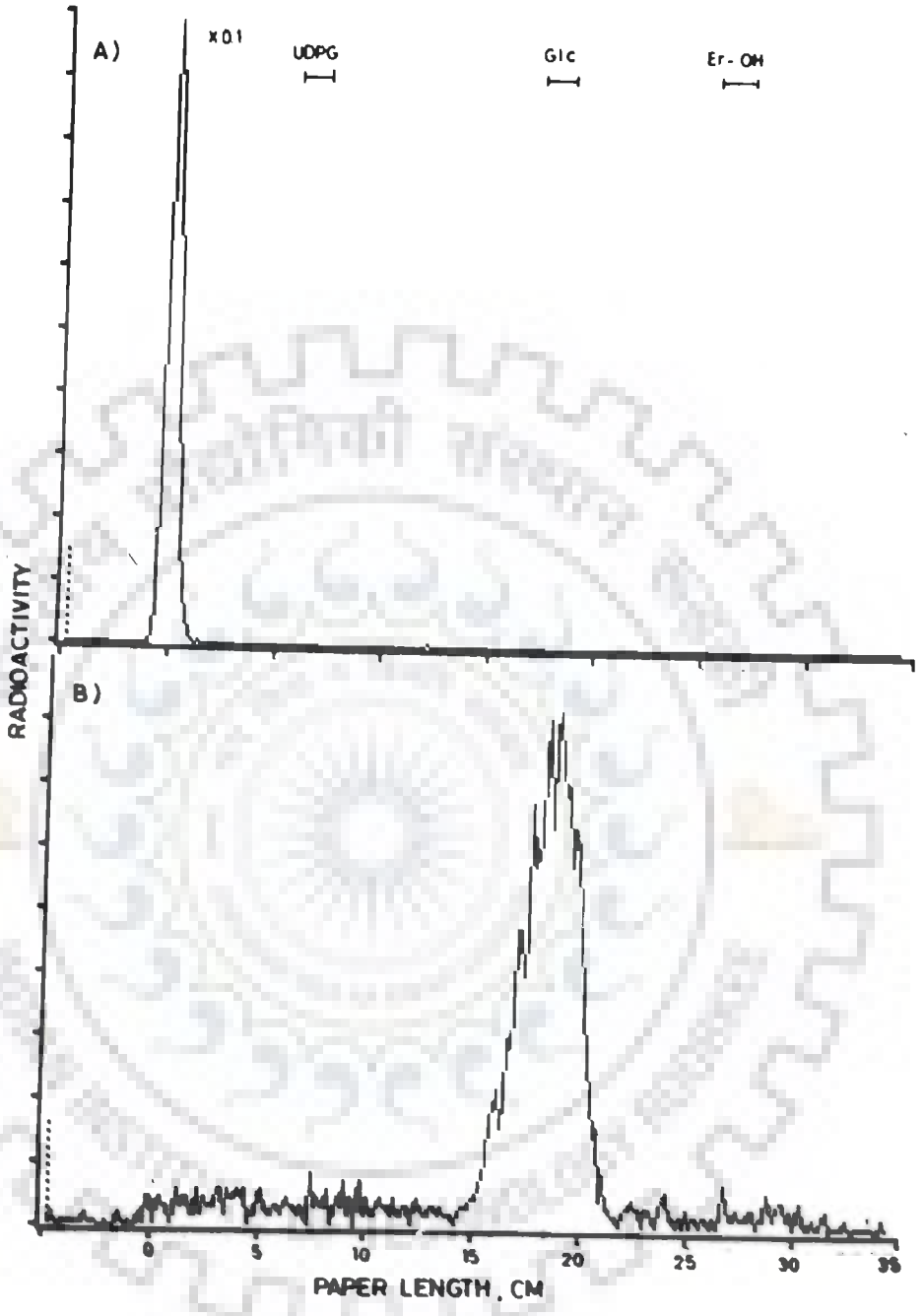
A)
x0.1
UDPG
Glc
Er-OH
B)
RADIOACTIVITY
0
5
10
15
20
25
30
35
PAPER LENGTH, CM

Fig. 17. B

reducing conditions yielded a value of 48K (Fig. 12.A, B). Since native-PAGE (Fig. 13) also showed a single protein band, GS-IIA is, in all probability, a single polypeptide of 48K molecular mass.

4.6.2 pH optima

The optimum pH for GS-IIA was found to be in the range of 7.4-7.6 (Fig. 18). The enzyme activity below and above this pH range was greatly reduced. Thus, in all experiments the enzyme activity was assayed at 7.4, unless stated otherwise.

4.6.3 Time course

The time course for the purified GS-IIA under the standard assay conditions, is shown in Fig. 19. It can be seen that the enzyme activity was linear upto 15-20 min and thereafter began to level off. Thus, for kinetic studies 15-20 min incubation period was used to ensure the initial rate of reaction.

4.6.4 Effect of some divalent metal ions

Table V shows the effect of Ca^{2+}, Mn^{2+} and Zn^{2+} on the activity of the purified GS-IIA under standard assay conditions. It was found that both EDTA and EGTA greatly ihibited the activity of enzyme, indicating that the enzyme required metal ions for its maximal activity. Since EGTA is a specific chelating agent for Ca^{2+}, the requirement of the enzyme for Ca^{2+} was quite obvious. This was further supported by the fact that by titrating the EGTA with Ca^{2+} to a slight excess of the latter, almost total activity (93% of the control) was restored. These results also suggest that the GS-IIA preparation had adequate amount of endogenous Ca^{2+} bound to the enzyme for its maximal activity and that the

Fig.18 pH OPTIMA OF GS-IIA

The enzyme activity was measured by the standard assay system except that the buffer and pH varied. The buffers used were acetate buffer, 50 mM, pH 5.0 -6.5; Tris-HCl 50 mM, pH 7.0-9.0.

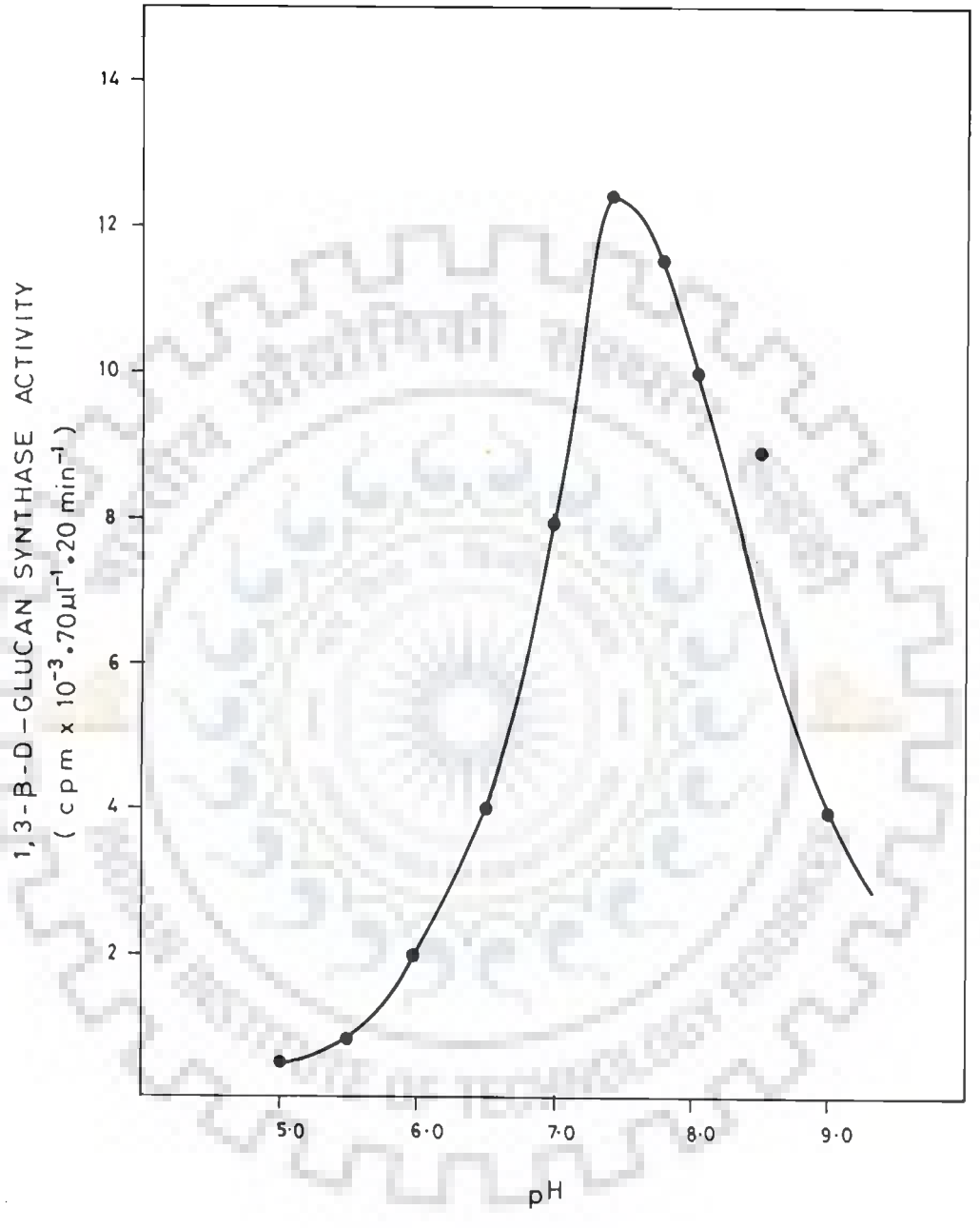
1,3-β-D-GLUCAN SYNTHASE ACTIVITY
(c p m x 10^{-3} .70µl^{-1}.20 min^{-1})
14
12
10
8
6
4
2
5·0
6·0
7·0
8·0
9·0
pH

Fig. 18

Fig.19 TIME COURSE OF GS-IIA

The enzyme activity was measured by the standard assay system as described under Materials and Methods except that the time period of incubation varied from 5 min to 30 min.

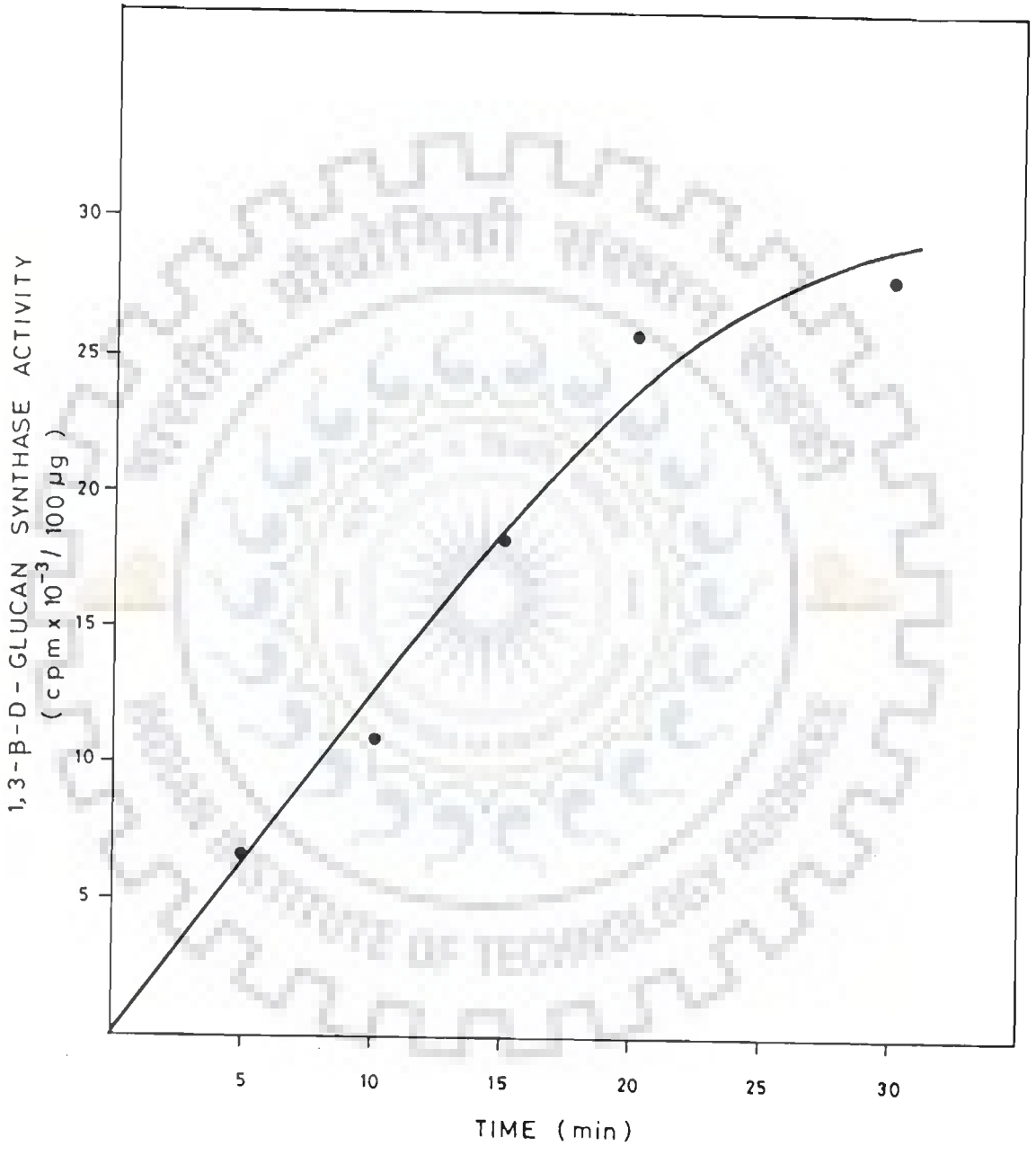
1,3-β-D-GLUCAN SYNTHASE ACTIVITY
(cpm x 10^{-3} / 100 µg)
30
25
20
15
10
5
5
10
15
20
25
30
TIME (min)

Fig. 19

TABLE V

EFFECT OF SOME DIVALENT METAL IONS ON ACTIVITY OF GS-IIA

The activity of the purified GS-IIA (low molecular mass form) was determined without and with indicated amounts of the metal ions using standard assay conditions as described under Materials and Methods.

Experiment	Activity of GS-IIA	
	Radioactivity incorporated	Per cent of control
	cpm	%
GS-IIA (Control)	13480	100
GS-IIA + EDTA,2 mM	5571	41.3
GS-IIA+EGTA[a], 2 mM	2938	21.8
GS-IIA+EGTA[a], 2 mM +Ca^{2+},10mM	12617	93.6
GS-IIA+Zn^{2+}, 1mM	4044	30.0
GS-IIA + Mn^{2+}, 1mM	7050	52.3

[a] EGTA = ethylene glycol bis (2-aminoethyl ether) N,N,N'N'-tetraacetate

Ca^{2+} ions were not removed during the purification steps which included dialysis and ion-exchange gel filtration steps. In contrast Zn^{2+} and Mn^{2+} inhibited the GS-IIA activity in vitro.

4.6.5 Kinetic parameters

The Km and Vmax values of GS-IIA for UDP-glucose substrate, as determined by the Lineweaver-Burk plot of 1/v vs 1/[S] (Fig. 20), were found to be 0.67 mM and 6.25 μmole. $min^{-1}.mg^{-1}$ protein, respectively.

4.6.6 Effect of some nucleotides and related compounds

The effects of different nucleotides and some other related compounds on the activity of the purified GS-IIA are shown in Table VI. The enzyme activity was inhibited from 40 to 50% by ATP, GTP, CTP, UDP and UMP at 5 mM concentration of the nucleotides. Interestingly, the extent of inhibition in all cases is nearly comparable. However, among the 5-nucleoside diphosphates and 5'-nucleoside monophosphates, only the UDP and UMP inhibited the enzyme activity by 40-45% whereas inhibitory effect of ADP, GDP, AMP or GMP was only marginal, varying from 20% to about 11%. Similarly, adenosine, adenine, D-ribose, glucose-6-phosphate, glucose-1-phosphate, dolichyl phosphate (dol-P) had no significant effect on the activity of the enzyme. Orthophosphate showed about 25% inhibition of the GS-IIA activity. From these results it appears that the phosphate group, especially the r-phosphate group in nucleoside triphosphates, is critical for the inhibitory action of nucleotides towards the activity of glucan

Fig.20 LINEWEAVER - BURK PLOT OF GS-IIA

The GS-IIA assay was carried out with varying amounts of non-radioactive donor substrate for 20 min with fixed amount of enzyme. Km and Vmax were computed from the intercepts of X - axis and Y - axis, respectively.

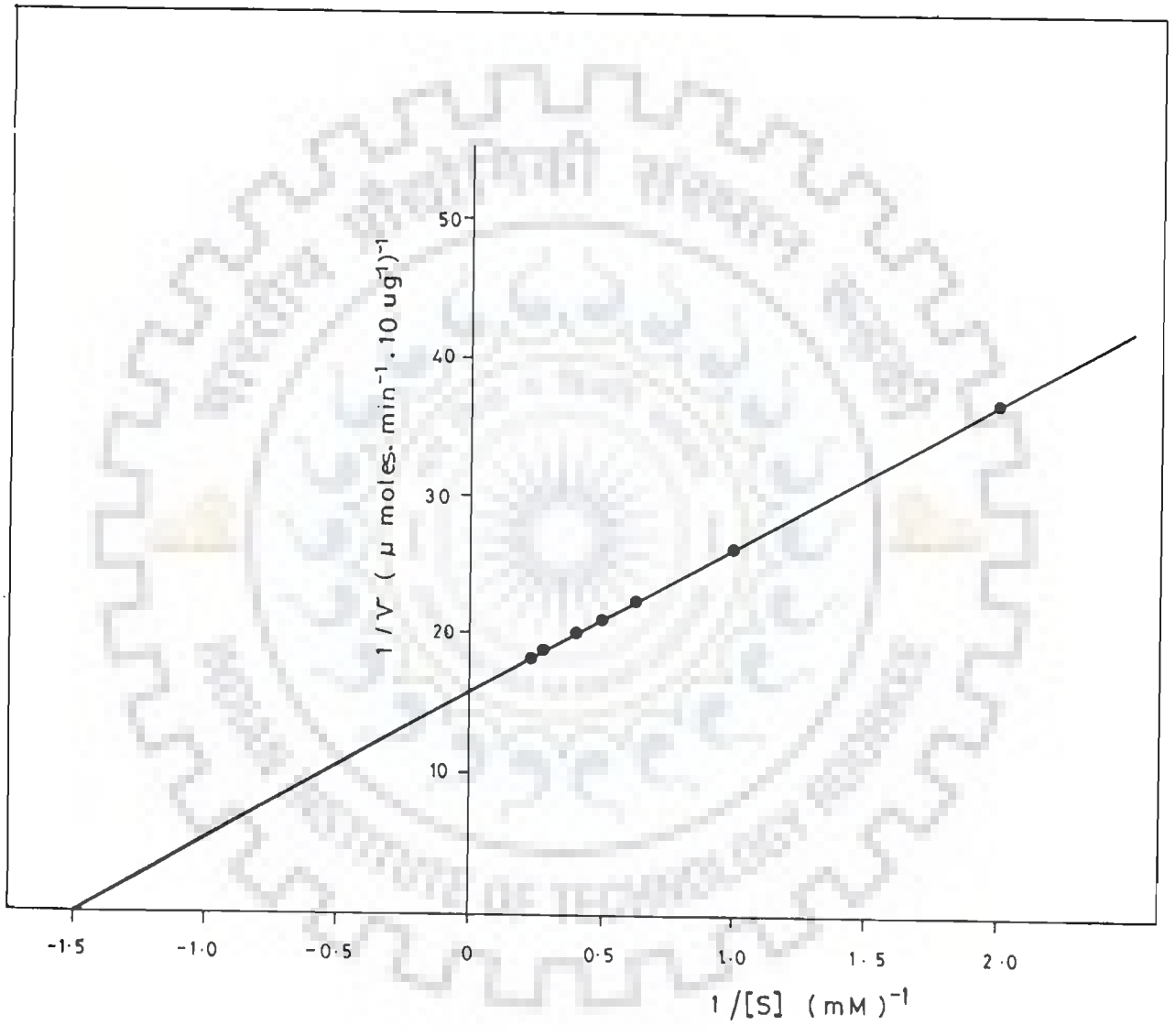

Fig. 20

TABLE VI

EFFECT OF SOME NUCLEOTIDES AND RELATED COMPOUNDS ON THE ACTIVITY OF GS-IIA.

Enzyme activity was assayed in the presence of 5mM nucleotides and related compounds as described under Materials and Methods.

Nucleotide added	Activity of GS-IIA	
	$nmole.min^{-1}$	%
None	7.0	100
ATP	3.6	51.4
GTP	4.2	60.0
CTP	3.8	54.2
ADP	5.9	84.2
GDP	5.6	80.0
UDP	3.9	55.7
AMP	6.0	85.7
GMP	6.2	88.57
UMP	4.3	61.4
Adenosine	6.8	97.0
Adenine	6.0	85.7
D-ribose	5.9	84.2
Glucose-1-PO_4	5.8	82.8
Glucose-6-PO_4	5.7	81.4
$KHPO_4$	5.3	75.7
Dolichyl phosphate	7.2	102.8

synthase-II. It may also be pointed out that dol-P, the carrier lipid of saccharides in the glycosylation of proteins in eukaryotic cells (Kornfeld and Kornfeld, 1985) also had no effect on the activity of the enzyme, suggesting, but by no means proving, that lipid-saccharide (dol-P-glucose) may not be involved in the biosynthesis of callose in which 1,3-β-D-glucan synthase is the principal enzyme involved.

4.6.7 Donor substrate specificity

GS-IIA and GS-IIB were found to be highly specific for UDP-glucose as the donor substrate in the in vitro formation of 1,3-β-D-glucan polymer (callose) and very little activity of the enzyme was observed when other sugar nucleotides such as GDP-[^{14}C] mannose UDP-[^{14}C] GlcNAc,UDP-[^{14}C] β-N-acetyl glucosamine or the lipid saccharides (dol-P-mannose, dol-P-glucose, dol-PP-GlcNAc and dol-PP-$GlcNAc_2$) were used as donor substrate (Table VII). It is of interest to note that dol-P-glucose which is a donor substrate for the terminal glucosyl residues of the lipid oligosaccharide, Glc_3-Man_9- $GlcNAc_2$-PP-dol, the immediate precursor of the asparagine-linked oligosaccharide chains of glycoproteins in all normal eukaryotic cells (Kornfeld and Kornfeld, 1985) was not the substrate for the GS-II enzyme. These results rule out the possibility of the involvement of lipid-saccharide intermediates, at least in vitro, in the biosynthesis of callose.

4.6.8 Effect of fluoride ions

Fluoride ions have been found to stimulate the purified GS-IIA from the peanut cotyledon plasma membranes (Table VIII). At 20

TABLE VII

DONOR SUBSTRATE SPECIFICITY

Substrate[a]	Conc	Activity of GS-IIA	Activity of GS-IIB
	cpm	cpm	cpm
UDP-[^{14}C] glucose	50000	12140	7051
GDP-[^{14}C] mannose	50000	993	1089
UDP-[^{14}C] GlcNAc	50000	701	505
Dol-P-[^{14}C] mannose	10000	175	169
Dol-P-[^{14}C] glucose	10000	180	170
Dol-PP-[^{14}C] GlcNAc	10000	198	179
Dol-P-[^{14}C] $GlcNAC_2$	10000	208	217

a. Lipid-saccharides were prepared by incubating yeast membranes with corresponding [^{14}C] -labeled sugar nucleotides as described by Sharma et al. (1982).

TABLE VIII

EFFECT OF FLUORIDE IONS ON THE ACTIVITY OF GS-IIA AND GS-IIB IN VITRO

The purified enzymes GS-IIA and GS-IIB were assayed in the presence and absence of fluoride ions using standard assay procedure.

Fluoride Conc.	Activity of GS-IIA	Percent of control	Activity of GS-IIB	Percent of control
(mM)	cpm	%	cpm	%
0	15672	100.0	11356	100.0
5	20359	129.9	10541	92.8
10	22915	146.2	11055	97.3
20	23684	151.1	11593	102.1

mM concentration of fluoride ions the stimulation of GS-IIA activity was aproximately 1.5 times. In this regard the peanut enzyme resembles the Candida albicans glucan synthase-II (Orlean and Ward, 1983), but seems to differ from the Saccharomyces cerevisiae enzyme as the latter required GTP (Larriba et al., 1981). In contrast, GS-IIB activity was not influenced by fluoride ions (Table VIII).

4.7 PROPERTIES OF GS-IIB

Since the purified GS-IIB fraction (after DEAE- Sephadex A-50 step) was extremely labile, some properties of the GS-IIB enzyme were also determined using partially purified fraction obtained from the second hydroxylapatite column (Fig. 9).

4.7.1 pH optima

The pH profile of the purified GS-IIB activity is shown in Fig. 21. The pH optima of the enzyme was found to be 7.0, which is slightly lower than the optimum pH (7.4-7.6) of GS-IIA (Fig 18). Infact, at pH 7.4 the activity of GS-IIB is only about 60% of the maximum. Similarly, the activity of GS-IIA at pH 7.0 was about two-third of the maximal activity at its optimum pH, pH 7.4. Whether this difference in optimum pH values of GS-IIA and GS-IIB is of any physiological significance, is not clear in the moment of time.

4.7.2 Molecular size

The molecular mass of the purified GS-IIB, enzyme as determined by the SDS-PAGE (Fig. 12.A), was found to be 57K which is significantly higher than the molecular mass of GS-IIA, 48K.

Fig.21 pH OPTIMA OF GS-IIB

The enzyme activity was measured by the standard assay system except that the buffer and pH varied. The buffers used were acetate buffer, 50 mM, pH 5.0 - 6.5; Tris-HCl, 50mM, pH 7.0 -9.0.

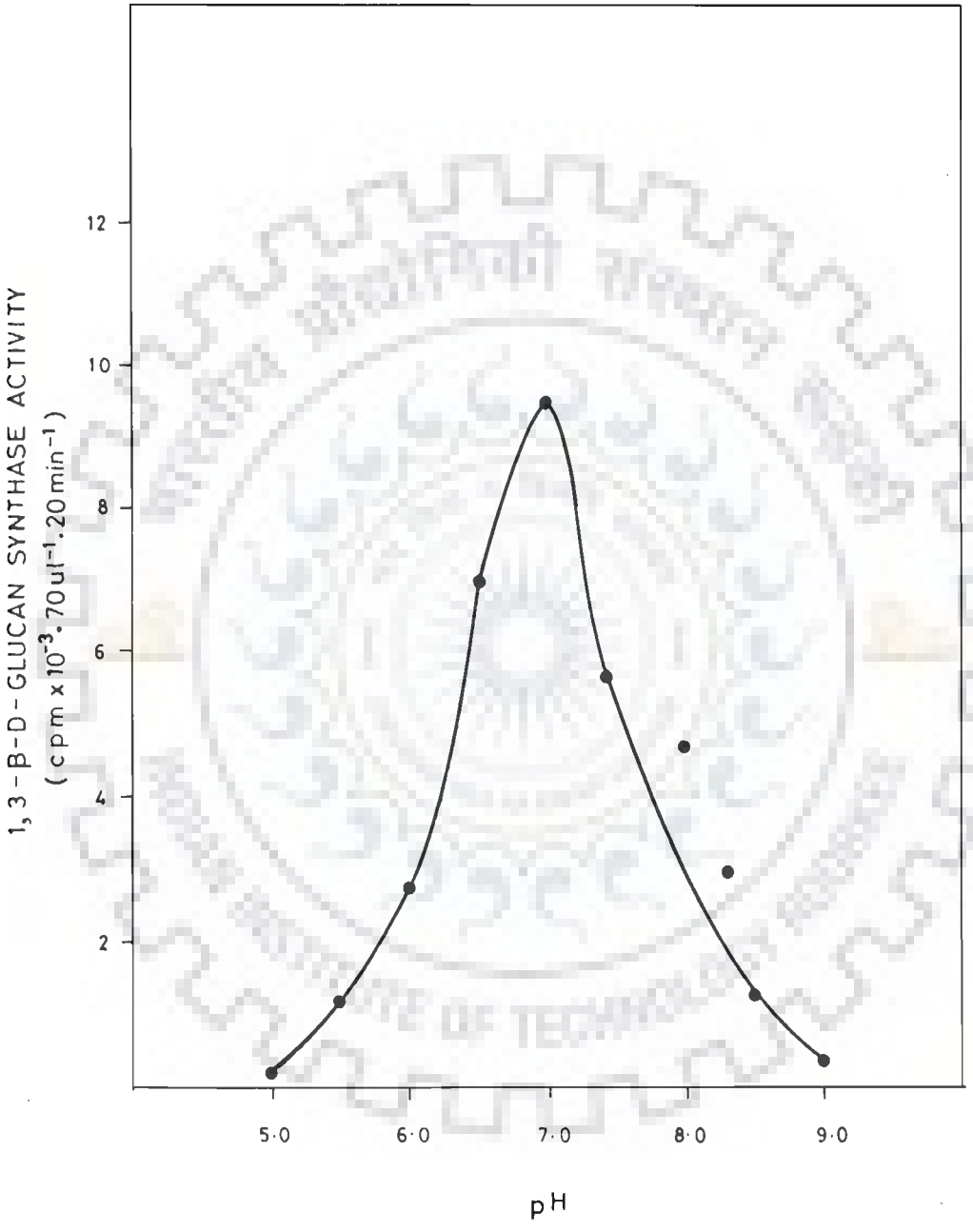
1, 3 - β-D-GLUCAN SYNTHASE ACTIVITY
(cpm x 10^{-3}. 70 ul^{-1}. 20 min^{-1})
12
10
8
6
4
2
5·0
6·0
7·0
8·0
9·0
pH

Fig. 21

4.7.3 Effect of some nucleotides and other compounds

The effect of some nucleotides and related compounds on the activity of GS-IIB is shown in Table IX. Like GS-IIA, the activity of GS-IIB enzyme was also inhibited by ATP, GTP, CTP, UDP and UMP, although the extent of inhibition of the latter was relatively smaller than the former.

4.7.4 Kinetic parameters

Fig. 22.A shows the effect of substrate (UDP-glucose) concentration on the velocity of the GS-IIB catalyzed reaction. As can be seen, the plot between velocity vs. substrate concentration is not hyperbolic and likewise double reciprocal plot of 1/v vs 1/[S] (Lineweaver-Burk plot, Fig. 22.B) is also not linear. These results indicate that reaction kinetics do not follow the Michaelis-Menten equation. At higher concentration of UDP-glucose (above 2.5 mM) the reaction rate increases significantly, indicating the substrate stimulation of GS-IIB activity. The same is also clearly shown by the Lineweaver-Burk plot (Fig. 22.B). The Km and Vmax values, obtained by extrapolating the linear portion of the Lineweaver-Burk plot, were found to be approximately 14.3 mM and 8.3 μ moles.min^{-1}.mg^{-1} protein, respectively. Although Km and Vmax values thus obtained are not correct estimates, there is a clear indication of the high substrate requirement of GS-IIB making the enzyme action physiologically unfavourable under normal physiological conditions. However, <u>in vivo</u>, there may be present an activator to make GS-IIB physiologically favourable.

TABLE IX

EFFECT OF SOME NUCLEOTIDES AND RELATED COMPOUNDS ON THE ACTIVITY OF GS-IIB.

Enzyme activity was assayed in the presence of 5mM nucleotides and related components as described under Materials and Methods.

Nucleotide added	Activity of GS-IIB	
	$nmole.min^{-1}$	%
None	5.0	100
ATP	3.0	60
GTP	3.5	70
CTP	3.4	68
ADP	4.0	80
GDP	3.8	76
UDP	3.2	64
AMP	4.3	86
GMP	4.4	88
UMP	3.1	62
Adenosine	4.5	90
Adenine	4.2	84
D-ribose	4.1	82
Glucose-1-PO_4	4.0	80
Glucose-6-PO_4	4.1	82
$KHPO_4$	3.7	74

Fig.22.A EFFECT OF SUBSTRATE (UDP-GLUCOSE) ON GS-IIB

The GS-IIB assay was caried out with varying amounts of non-radioactive donor substrate for 20 min with fixed amount of enzyme. The X-axis represents the UDP-glucose (mM) concentration. The Y - axis represents the velocity (n mole.30 $\mu g^{-1}.min^{-1}$)

Fig.22.B LINEWEAVER-BURK PLOT OF GS-IIB

Km and Vmax were obtained by extrapolating the linear portion of the double - reciprocal plot on the X-axis and Y-axis, respectively.

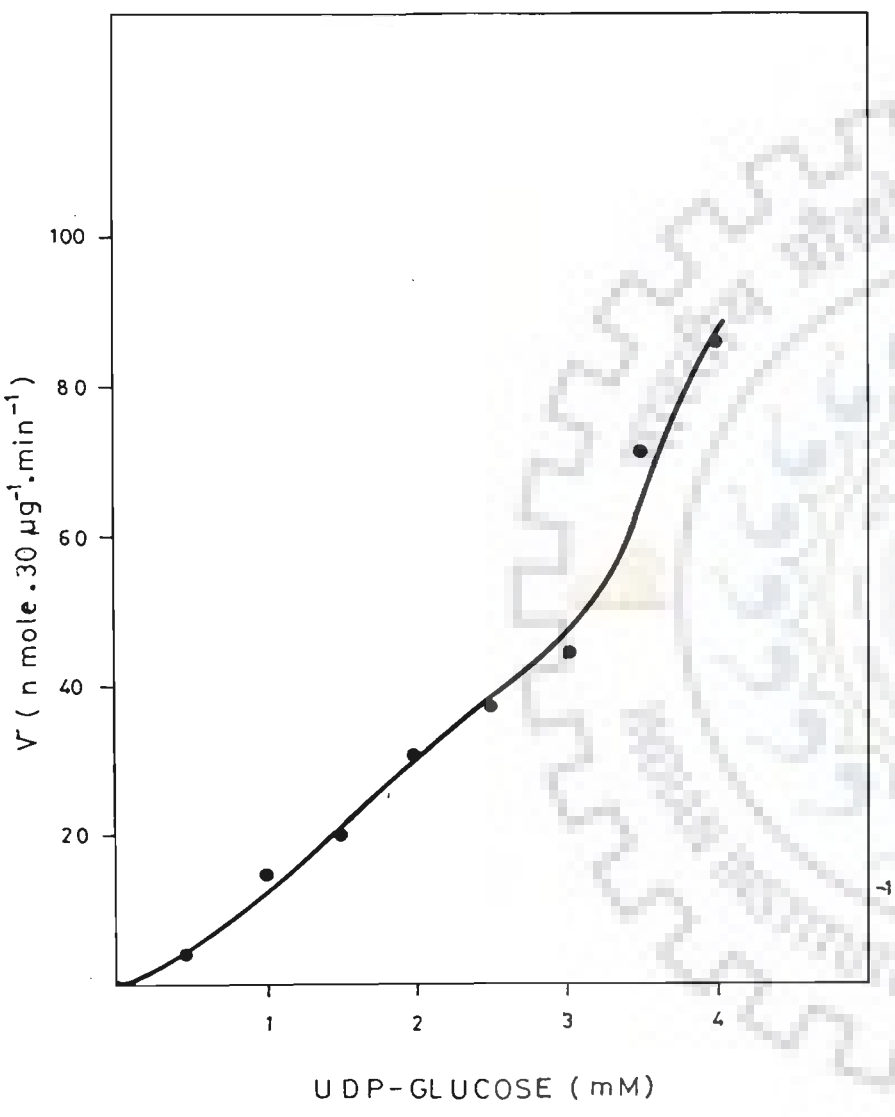

Fig. 22.A

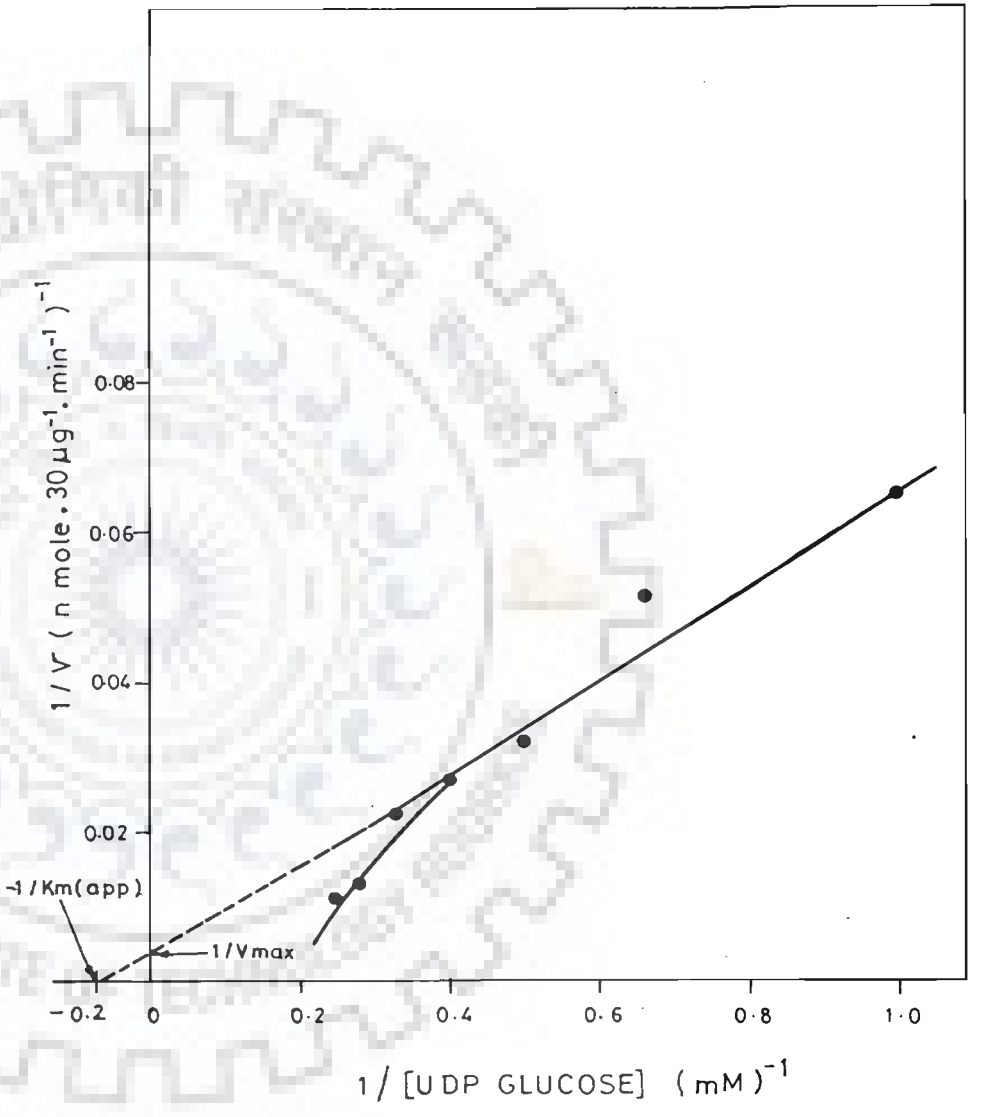

Fig.22.B

In the moment of time, we do not have any evidence to support this view. In view of the above, GS-IIA appears to be the main 1,3-β-D-glucan synthase involved in the biosynthesis of callose.

4.8 KINETICS OF INHIBITION

Since, on the basis of kinetic data GS-IIA enzyme appears to be physiologically important, the nature of inhibition of ATP, GTP and CTP was investigated. The Lineweaver-Burk plots with and without the fixed concentration of nucleotide (Fig. 23) show that the inhibition by ATP and CTP was of the competitive type, but for GTP it was of noncompetitive type with Ki values of 1.6, 1.0 and 2.6 mM, respectively. These values are comparable with the Km values of the substrate, UDP-glucose for the enzyme. However, further work will be necessary before a definite role of the nucleotides in the regulation of 1,3-β-D-glucan synthase activity can be envisaged in vivo.

4.9 COMPARISON OF PROPERTIES OF GS-IIA AND GS-IIB

Table X shows the comparison between the properties of the purified GS-IIA and GS-IIB of peanut cotyledons. The two enzymes show characteristic difference in their properties. For instance, besides having different pH optima, Km and Vmax values, molecular masses, H_2N-terminal amino acid and stability, the two enzymes are strikingly different in their immunological responses. While GS-IIA showed immunogenic property, GS-IIB was found to be nonimmunogenic rabbits. In addition, the polyclonal monospecific antibodies made against GS-IIA protein did not cross-react with GS-IIB. Although data given in Table III clearly shows that GS-IIA and GS-IIB are different enzymes, the

Fig.23 LINEWEAVER - BURK PLOT SHOWING THE NATURE OF INHIBITION OF ATP, GTP AND CTP ON GS-IIA

The GS-IIA assay was carried out with varying amounts of non-radioactive donor substrate for 20 min with fixed amount of enzyme, (●——●). The enzyme was also assayed in the presence of 1mM CTP (○——○); 1mM ATP (△——△) and 1 mM GTP (■——■).

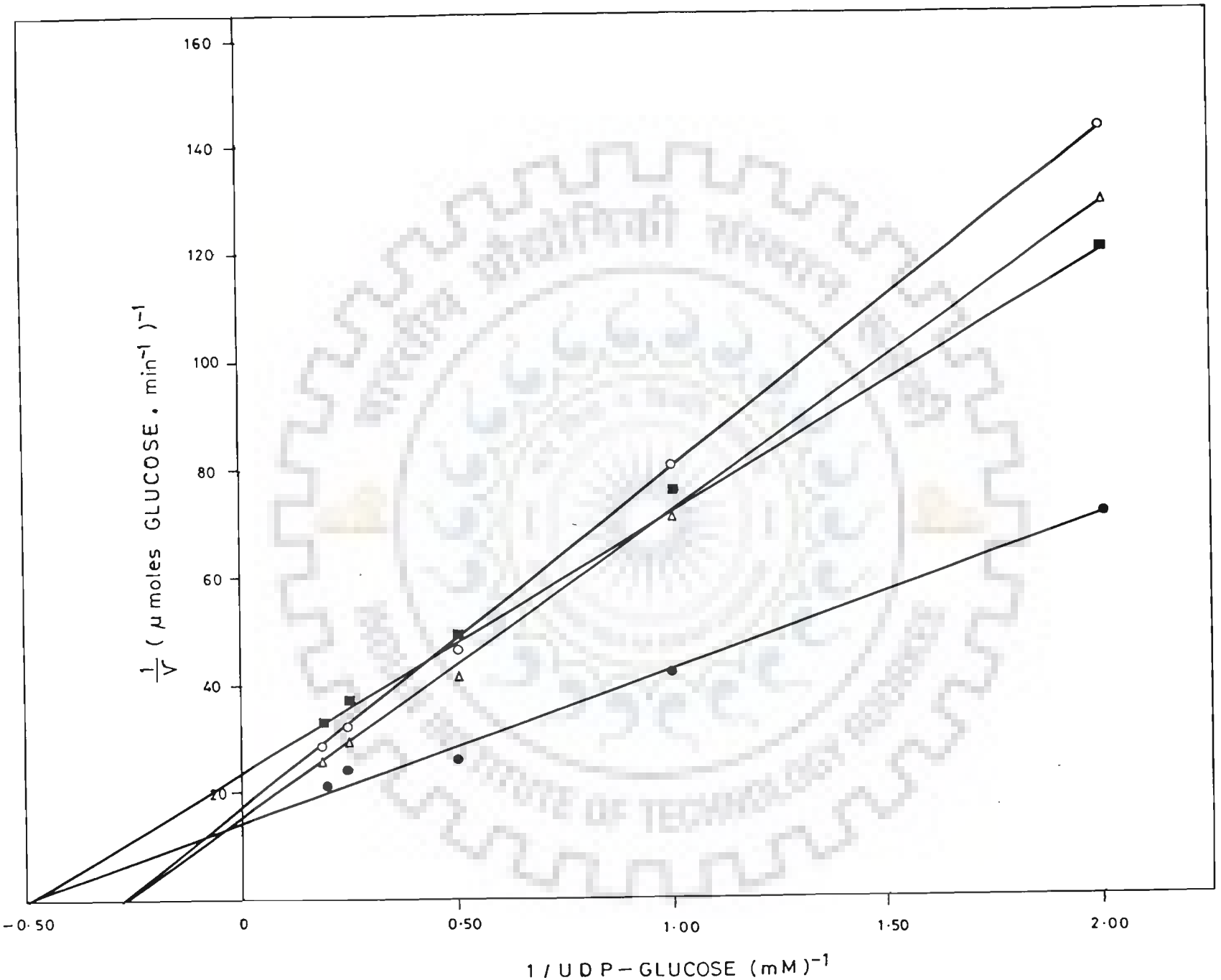
160
140
120
100
80
60
40
20
$\frac{1}{V}$ (μ moles GLUCOSE . min^{-1})$^{-1}$
-0·50
0
0·50
1·00
1·50
2·00
1 / UDP – GLUCOSE (mM)$^{-1}$

Fig. 23

TABLE X

A COMPARISON OF PROPERTIES OF PURIFIED GS-IIA AND GS-IIB FROM PLASMA MEMBRANE FRACTION OF PEANUT COTYLEDONS

Properties	GS-IIA	GS-IIB
Molecular weight	48 K	57 K
Subunits present	Single polypeptide	Single polypeptide
pH optima	7.4-7.6	7.0
Metal ion requirement	Ca^{2+}, 2-5 mM	Ca^{2+}, 5 mM
Km	0.67 mM	14.3 mM
Vmax	6.25 μmole.min^{-1}.mg^{-1}	8.3 μmole.min^{-1}.mg^{-1}
Inhibition by 5 mM,		
ATP	49.6	40
GTP	40.0	30
CTP	45.8	32
UDP	44.3	36
UMP	38.6	38
Activation by		
Fluoride(20 mM)	50%	None
Stability at -20°C	Stable	Unstable
Elution from hydroxylapatite column I	95 mM, PO_4^{3-} gradient	220 mM, PO_4^{3-} gradient
Substrate specificity	Highly specific for UDP-Glucose	Highly specific for UDP-Glucose
Immunogenicity	Yes	No
H_2N-terminal	Leu	Lys

structural relationship between the two enzymes cannot be completely ruled out. For instance, it is not clear to us if the GS-IIA (low molecular mass form) was not a product of protease action on GS-IIB (higher molecular form). The work is in progress in this direction.

5.0 DISCUSSION

The present study describes the purification to apparent homogeneity and characterization of two forms of 1,3-β-D-glucan synthase, commonly known as glucan synthase- II (GS-II). Essentially, all higher plants contain UDP-glucose : 1,3-β-D-glucan synthase. This enzyme is largely found on the plasma membrane, and in most cases is latent and only becomes activated by perturbed conditions, such as mechanical injury or attack by a pathogen (McNairn, 1972; Eschrich, 1975; Aist, 1976; Vithanage and Knox, 1977; Jaffe et al., 1983 and Kauss, 1987). Consequently, there is a rapid deposition of callose (a 1,3-β-D-glucan polymer) in vivo, probably outside the plasma membrane, as a part of the repair or the defence mechanism against the pathogen attack (Fig. 24). Recently GS-II has also been recognized as the target enzyme for Ca^{2+} (Kauss, 1987). As far as we are aware this enzyme has so far not been purified to homogeneity from any plant tissue, although a number of workers reported the partial purification of the enzyme from a few plant sources (Wasserman and McCarthy, 1986; Eiberger and Wasserman, 1987; Read and Delmer, 1987; Lawson et al., 1989). Recently, Fink et al. (1990) have purified the enzyme 12-fold from the suspension-cell culture of Glycine max and made polyclonal antibody against a 31K polypeptide band which was enriched during solubilization by digitonin followed by linear sucrose density gradients. This is an interesting piece of information, but direct evidence for the enzyme activity in the 31K protein is yet to be obtained. Hence, the purification scheme presented in the thesis is the report that provides the purification of

Fig.24 DEFENCE MECHANISM AGAINST PATHOGEN ATTACK.

Induction of callose as part of plant defence responses against a fungal pathogen. Symbols denote : R, putative elicitor receptor; S, signal transduction which may include Ca^{2+} or other elements ; N, nucleus ; C, callose; 1,3-β-GS, 1,3-β-Glucan synthase; A, amino acid uptake. (Modified from; Kauss, 1985).

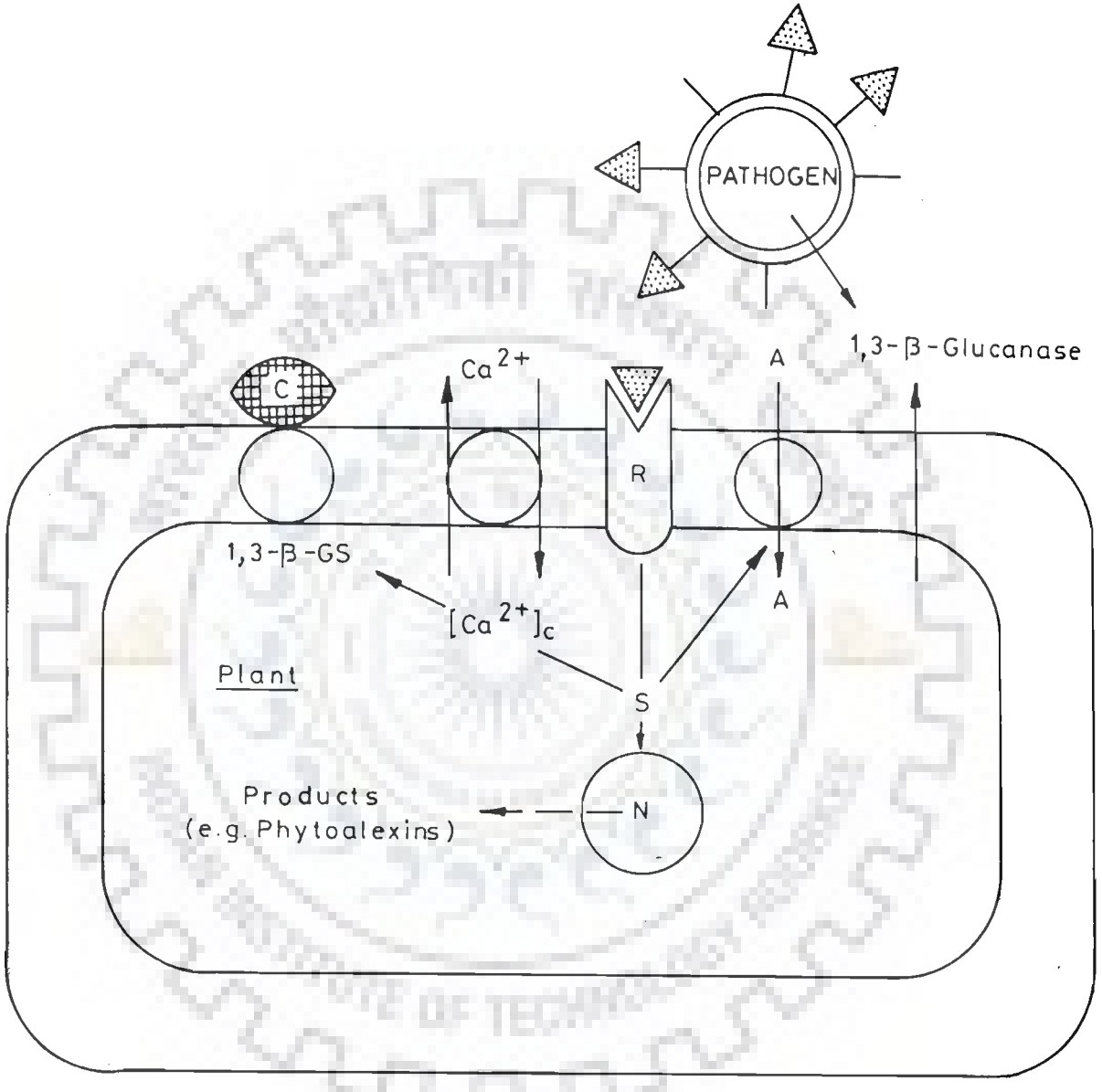
PATHOGEN
1,3-β-Glucanase
C
Ca 2+
A
R
1,3-β-GS
A
[Ca 2+]c
Plant
S
Products
(e.g. Phytoalexins)
N

Fig. 24

GS-II to homogeneity. The purification scheme is relatively simple and involved the use of conventional methods of purification of membrane proteins namely, preparation of high specific activity PM fraction, selective solubilization of the membrane-bound enzyme with digitonin, linear density gradient fractionation and chromatography on hydroxylapatite and DEAE-sephadex A-50 columns. The two forms of GS-II (GS-IIA and GS-IIB) were homogeneous as judged by SDS-PAGE, immunological specificity and H_2N-terminal amino acid determinations.

The 6-7 day old germinating peanut cotyledons provided a very suitable source for the preparation of high specific activity PM fraction, as at this physiological state not only the total activity, but also the specific activity of GS-II was maximal. The PM fraction obtained following the method described by Sharma et al. (1986) was of high purity and was free from the cross-contamination of other endomembrane fractions such as Golgi apparatus as judged by the distribution of activity of marker enzymes for PM, ER and GA (Ray, 1977; Hall, 1983; Green, 1983). Since, PM fraction was obtained from the microsomal fraction prepared from the cotyledons in the presence of EDTA and without Mg^{2+}, it was also considered free from the rough endoplasmic reticulum (rER), a common contaminant of PM (Hall, 1983).

The enzyme is tightly bound to the membrane and could be released by detergent. Digitonin and CHAPS were found to be the most effective detergents and GS-II activity was best solubilized (released in the supernatant fraction after 130,000 xg

centrifugation) by 0.5% (w/v) digitonin (final concentration) at a protein-to-detergent ratio of 1:6 in the presence of 250 mM sucrose. The latter was necessary for stabilizing the detergent solubilized enzyme. Other nonionic detergents used, namely Triton X-100, Nonidet P-40 and octylglucoside inactivated the enzyme and as such were unsuitable for solubilization of GS-II. In the case of Triton X - 100 solubilized enzyme, Wasserman and McCarthy (1986) reported the depletion of about 90% of total membrane phospholipids resulting in reduction of glucan synthase-II activity by 80-90% and addition of phospholipids restored the activity. Thus, it appears that Triton X-100 and Nonidet P-40 removed the phospholipids from the membrane needed for the enzyme activity, whereas digitonin and CHAPS did not do so. These results clearly indicated the specificity of the interaction of PM-bound GS-II with the digitonin. In this regard the behaviour of peanut cotyledon PM GS-II towards nonionic detergents resembles the enzymes of other plants (Tsai and Hassid, 1971; Heiniger, 1983; Henry and Stone, 1983; Eiberger et al., 1985; Wasserman and McCarthy, 1986; Eiberger and Wasserman, 1987) except that greatly varying concentrations (0.1-16%) of digitonin were used to solubilize the enzyme activity from PM.

The digitonin-solubilized enzyme exhibited 1.5-2.8-fold increase in the total and specific activity compared to the membrane-bound enzyme, clearly indicating the activation and selective solubilization of the enzyme. The mechanism by which digitonin activates the GS-II activity is not known. However, Wasserman

and McCarthy (1986) believed that the digitonin extracted enzyme contained a layer of attached `boundary lipid', as has been shown in the case of gastric H,K-ATPase (Olaisson et al., 1985), which was responsible for the enzyme activation.

Normally, the detergent treatment destroys the hydrophobic environment of the membrane structure and releases the proteins into a more hydrophillic medium. Surprisingly, this change in the natural environment by digitonin did not render the enzyme inactive, instead the enzyme was activated. Obviously, some stabilizing factors such as phospholipids were released from the PM along with GS-II which directly selectively interacted with the enzyme in solution. Such a concept will be in line with the formation of boundary lipid as proposed by Wasserman and McCarthy (1986). Since the activity of the solubilized enzyme was greatly enhanced by digitonin, it is tempting to assume that the loss of essential phospholipids or disintegration of subunits from the holoenzyme has not occurred during the digitonin solubilization of the enzyme.

The purification of the detergent solubilized enzymes is always a difficult task, especially when the critical micelle concentration (CMC) of digitonin is only 0.02% (Sloan et al., 1987) which is much lower than 0.5% or 0.1% concentrations of digitonin used either for the solubilization of the enzyme or in the elution and incubation buffers, respectively. Fortunately, in the presence of 250 mM sucrose the solubilized enzyme is fairly stable at 0-4^{o}C for 15 days with only 25 % loss of activity (Fig. 5). However, at room temperature (25^{o}C) about

two-third of the enzyme activity was lost in 24 h.

One of the widely used techniques used for purifying the membrane-bound enzymes is density gradient centrifugation (Addison and Scarborough, 1981; Bowman et al., 1981; Smith and Scarborough, 1984; Mandala and Taiz, 1985; Kasamo, 1986; Eiberger and Wasserman, 1987; Fink et al., 1990). In the present purification scheme also this step was found very useful as a 2-fold step purification was achieved. The enzyme peak (Fig. 6) was not quite symmetrical but appeared to have another overlapping peak on the left of the major peak. These results suggested the presence of another enzyme with little higher but not too high molecular mass to be fully separated. In fact, this observation was found to be true when to our surprise two forms of GS-II (GS-IIA and GS-IIB) corresponding to molecular mass of 48K and 57K were separated on hydroxylapatite column (Fig. 7). The multiplicity of soluble glucan synthase activity in spinach leaves has been demonstrated by Tacke et al. (1991).

The hydroxylapatite column chromatography not only yielded a substantial purification, but it also resolved the two forms of GS-II designated as GS-IIA and GS-IIB, eluting at about 95 mM and 220 mM phosphate gradients. This was an unexpected observation. The molecular masses of GS-IIA and GS-IIB fractions were found to be 48K and 57K. The question that now arises is the following. Has GS-IIA (lower molecular mass form) originated from GS-IIB, (higher molecular mass form) as a result of specific proteolytic cleavage ? This question is also relevant because proteolytic

activations of GS-II have been reported (Kauss et al., 1983) and the specific activity of GS-IIA is definitely higher than the specific activity of GS-IIB (Table III) and the former is also more stable than the latter. However, a comparison of properties (Table X) and the fact that the ratio of GS-IIA to GS-IIB (Table XI) in the digitonin solubilized enzyme did not change with storage, suggest but by no means prove, that GS-IIA and GS-IIB are two different enzymes. If GS-IIA were to originate from GS-IIB as a result of proteolytic action, then it would be expected that the relative concentration of GS-IIA should increase with a concomitant decrease in the relative concentration of the high molecular mass form, GS-IIB. These observations were further supported by the immunological responses of the two enzymes. The polyclonal monospecific antibodies made against GS-IIA did not at all cross-react with GS-IIB. However, inspite of more points favouring the different origins of GS-IIA and GS-IIB, the possibility that the two forms of glucan synthase-II are structurally related cannot be totally ruled out in the moment of time. Since, we have purified these enzymes to an apparent homogeneity, studies relating to the structural relationship, etc. can now be undertaken.

The polysaccharide formed during the reaction by the purified glucosyl transferase was not attacked by α- and β-amylase, but was completely hydrolyzed by a β-(1,3)-glucanase. These results indicated the absence of glycogen formation and presence of β-(1,3)-linked glucosyl residues in the reaction product (Shematek et al., 1980; Orlean, 1982). The presence of β-(1,3)-linkage was

TABLE XI

EFFECT OF STORAGE ON THE RELATIVE CONCENTRATION OF GS-IIA AND GS-IIB IN THE DIGITONIN EXTRACT

The digitonin-solubilized enzyme (130,000 x g supernatant fraction) was stored at -20°C for the indicated periods before subjecting to the hydroxylapatite column for separation of GS-IIA and GS-IIB fractions. Enzyme activities were determined using standard assay procedure.

Period of storage	Total enzyme activity		Ratio
	GS-IIA	GS-IIB	GS-IIA/GS-IIB
days	$nmole.min^{-1}$	$nmole.min^{-1}$	
2	229.4	151.4	1.51
3	208.3	147.5	1.41
4	291.6	185.5	1.57
5	299.0	191.1	1.56

further confirmed by the total resistance of the polysaccharide to periodate treatment, since the total radioactivity released after periodate oxidation, followed by reduction and acid hydrolysis travelled with glucose. However, amylose and glycogen were completely degraded by this treatment. In addition, absence of radioactive erythritol showed that there were no detectable 1,4-glucosidic linkages in the reaction products (Orlean, 1982). Thus, the polysaccharide formed by the GS-IIA or GS-IIB under the reaction conditions used appeared to be a linear β-(1,3)-linked glucan. In this respect the peanut cotyledon plasma membrane enzyme resembles that of the yeast glucan synthase (Shematek et al., 1980; Orlean, 1982). These results are also consistent with the characteristic observation that the isolated plant plasma membranes largely synthesize 1,3-β-D-glucan and their ability to synthesize 1,4-β-D-glucan polymer from the external UDP-glucose is lost (Delmer, 1987). In contrast, the plasma membrane-located 1,3-β-D-glucan synthase becomes active on cell homogenization which otherwise was fully latent in intact cells (Fink et al., 1990). Callose is used in plants to plug wounds and it could be readily removed (Northcote, 1985).

The possibility that a very small number of 1,4-β-linkages remained undetected cannot be excluded completely as the plant membranes do incorporate glucose from UDP-glucose into xyloglucan in vitro (Gordon and Mclachlan, 1989). Furthermore, Jacob and Northcote (1985) and Delmer (1987) strongly believe that cellulose synthase (1,4-β-D-glucan synthase, GS-I) and the callose synthase (1,3-β-D-glucan synthase, GS-II) belong to the

same enzyme complex which may change its specificity on perturbation of PM. According to the model recently proposed by Delmer (1987), there is only one plasma membrane associated UDP-glucose: glucosyltransferase that is capable of catalyzing the synthesis of cellulose or callose. In the event of cellulose synthesis, as in the case of intact cells, a 18K polypeptide is associated with the glucosyltransferase when the intracellular level of Ca^{2+} is low. Under these conditions the enzyme preferentially transfers glucose to 4-OH of the terminal glucose to the growing glucan chain. But, in the damaged cells or isolated PM 18K polypeptide was dissociated from the enzyme presumably because of the elevated intracellular level of Ca^{2+} which is also reported to inhibit the cellulose synthase activity (Delmer et al., 1982). Thus, in intact cells with low levels of free Ca^{2+}, the callose synthase (GS-II) will be latent. Although the model of Delmer is still to be proved, it does provide an attractive explanation for the loss of cellulose synthesizing ability of isolated PM.

Now, with the purification to apparent homogeneity of GS-II described here, it should be possible to test this hypothesis. Stimulation of 1,3-β-D-glucan synthase by micromolar levels of Ca^{2+} using different plant sources has been shown (Kauss et al., 1983; Delmer et al., 1984; Blaschek et al., 1985; Kohle et al., 1985; Morrow and Lucas, 1986; Kauss, 1987). Recently, Hayashi et al. (1987) have shown that micromolar concentration of Ca^{2+} act by raising the Vmax as well as increasing affinity for UDP-glucose. It is to note that the limited proteolysis by trypsin

in the presence of digitonin of the soybean cell 1,3-β-D-glucan synthase rendered it insensitive to Ca^{2+} stimulation (Kauss et al., 1983). These workers also found that both Ca^{2+} - stimulated native enzyme as well as the trypsinized preparation were inhibited to about the same extent by calmidazolium and trifluoperazine. Since these drugs bind to calmodulin only in presence of Ca^{2+}, the mediation by calmodulin in Ca^{2+} stimulation of 1,3-β-D-glucan synthase seems to be unlikely. The peanut cotyledon GS-IIA is also stimulated by NaF. Thus, it resembles the β-glucan synthase from Candida albicans (Orlean and Ward, 1983). The mechanism of NaF activation of peanut cotyledon GS-IIA is not fully understood. However, it does not seem to involve the inhibition of nucleotidases or phosphatases as has been implicated in the case of Saccharomyces cerevisiae 1,3-β-D-glucan synthase (Larriba et al., 1981), since activation of the peanut enzyme is independent of nucleotides.

The activity of the purified GS-II was greatly stimulated by Ca^{2+}. Kohle et al. (1985) have proposed a direct and reversible action of Ca^{2+} ions without mediation of calmodulin. However, covalent modification of 1,3-β-D-glucan synthase cannot be ruled out at the moment and would require further research in this direction. Surprisingly, GS-IIA was inhibited by Mn^{2+} and Zn^{2+}. The physiological significance of the inhibition of these divalent metal ions is not understood.

ATP and GTP have been reported to activate the yeast glucan synthase (Shematek et al., 1980; Shematek and Cabib, 1980; Orlean, 1982). In contrast, we have found that the peanut

cotyledons plasma membrane 1,3-β-D-glucan synthase as moderately inhibited by ATP, GTP and CTP indicating that the yeast and the peanut plasma membrane glucan synthases are different and that in the latter case the regulatory mechanism is likely to be different from the one proposed for yeast glucan synthase by Shematek and Cabib (1980). Since ADP, GDP, AMP and GMP also inhibit the purified GS-IIA and GS-IIB enzyme, the nucleotides may play an important role in the regulation of 1,3-β-glucan synthesis. In respect to the inhibition of GS-II activity by nucleotides, the peanut enzyme resembles the enzyme from Saprolegnia (Fevre, 1983).

The molecular masses of the purified GS-IIA and GS-IIB polypeptides as determined by SDS-PAGE were found to be 48K and 57K, respectively. Since, except this report the glucan synthase-II has not been purified to homogeneity so far, the comparison of the molecular masses of GS-IIA and GS-IIB with the enzyme(s) from other plant sources was not possible. However, recently, Frost et al. (1990) using the photoaffinity probe 5-azidouridine 5'-[β-^{32}P] - diphosphate glucose ($5N_3[^{32}P]$ UDP-glucose) identified a 57K polypeptide which is likely to be the UDP-glucose-binding polypeptide of UDP-glucose: 1,3-β-D-glucan (callose) synthase from red beet storage tissue. More recently Dhugga and Ray (1991) have shown, using isoelectric focussing, that a 55K polypeptide was associated with 1,3-β-D-glucan synthase activity from the plasma membranes of pea. It is very interesting that the molecular mass of GS-IIB form of the glucan synthase from the PM of peanut cotyledons was found to

be 57K. In our opinion it is not a coincidence, but may represent a correct picture regarding the molecular masses of callose synthase. The GS-IIA with molecular mass of 48K may then represent the processed form of GS-IIB in peanut cotyledons, which in the case of red beet storage tissue was not present and therefore could not be labeled. Read and Delmer (1987) and Delmer and Soloman (1989) identified, by affinity labeling with UDP-[^{3}H] pyridoxal and [^{32}P] UDP-glucose, two polypeptides with molecular masses of about 34K and 50K, which they related to the GS-II activity of these two, however, only the 50K polypeptide co-purified with 1,3-β-D-glucan synthase activity in sucrose density gradient. It may also be pointed out here that the monoclonal antibody able to precipitate 1,3-β-D-glucan synthase and the protein thus precipitated gave a double band of 60-62K but this band was not enriched in the glucan synthase-II activity peak. Hence, from these results it is abundantly clear that indirect evidences obtained regarding the molecular mass of glucan synthase-II are only indicative and cannot be taken to represent the correct values. In this regard the results of the present study clearly established the molecular masses of GS-IIA and GS-IIB proteins containing single polypeptides as 48K and 57K, respectively. To a reasonable extent the values are in line with the molecular masses predicted by the affinity labeling and in an excellent agreement with 57K molecular mass obtained for the red beet enzyme by photoaffinity labeling (Frost et al., 1990). It would be interesting to see if the antibody made against GS-IIA also cross-reacted with the red beet enzyme.

6.0 SUMMARY

1,3-β-D-glucan synthase is the major enzyme which synthesizes callose, a stress-related polysaccharide. It is a plasma membrane located enzyme and is activated by mechanical perturbation or in defence when the plant is attacked by pathogens. In this report we have described the purification to homogeneity of two forms of 1,3-β-D-glucan synthase from the plasma membranes of germinating peanut cotyledons. The highly enriched plasma membrane fraction was obtained from peanut cotyledons using differential and sucrose density gradient centrifugation. The distribution of 1,3-β-D-glucan synthase activity among various endomembranes showed that the enzyme was largely localized in the plasma membranes. The physiological state of the seed was found to influence the level of 1,3-β-D-glucan synthase activity in the plasma membranes. In peanut cotyledons maximum enzyme activity was observed on the 7^{th} day of germination ($35^{o}C$). For instance, the activity of the enzyme decreased sharply from 13,000 $nmol.min^{-1}$ to 850 $nmol.min^{-1}$ in the next 24h following the peak activity. Similarly on the 4^{th} day the enzyme activity was 550 $nmol.min^{-1}$ compared to 13,000 $nmol.min^{-1}$ on 7^{th} day. The specific activity of the enzyme, however, was found to be highest between 4^{th} and 7^{th} day. Thus, in all our experiments unless stated otherwise 6-7 day old germinating peanut cotyledons were used for the preparation of plasma membrane.

In order to solubilize the membrane-bound 1,3-β-D-glucan synthase a number of detergents, including β-octylglucoside, Triton X-100,

Nonidet P-40, digitonin and CHAPS wre tried. Of these detergents digitonin and CHAPS were found to be suitable for solubilizing enzymes from the plasma membrane. Both these detergents were also found to stimulate the enzyme activity. In contrast, Nonidet P-40, Triton X-100 and β-octylglucoside were extremely inhibitory. Digitonin was therefore used to solulbilize the enzyme from plasma membrane. Optimum solubilization was obtained with 0.5% digitonin at a protein-to-detergent ratio of 1:6.

Purification was achieved using the conventional methods of protein purification. Initial purification of the enzyme was achieved by preparing highly enriched plasma membrane fraction which was free from cross-contamination of Golgi apparatus and endoplasmic reticulum. After this step the soluble enzyme was subjected to sucrose density gradient centrifugation. It was found that of the total enzyme activity applied on the sucrose gradient about two-third activity was concentrated in fraction numbers 6-9, corresponding to 46-34% sucrose gradient. After this step the purification of GS-II was approximately 620-fold. The above sucrose gradient peak fractions were pooled, dialyzed and applied to hydroxylapatite column. 1,3-β-D-glucan synthase activity was eluted in two distinct peaks (peak I and peak II) corresponding to 95 mM and 220 mM phosphate concentration. The enzyme activity in these peaks was referred to as GS-IIA and GS-IIB which had an overall purification of about 1912- and 1308-fold. The GS-IIA and GS-IIB were rechromatographed on a hydroxylapatite column to give an enhanced purification of 2351-fold and 2010 -fold respectively. The final purification step

involved DEAE sephadex column chromatography which increased the purification fold of GS-IIA to 6618-fold but GS-IIB proved to be labile and only one-third activity remained. On SDS-PAGE both the forms of enzymes moved as a single band corresponding to molecular weights of 48K and 57K, respectively. Polyclonal monospecific antibodies were made against the purified GS-IIA and GS-IIB enzyme preparations. GS-IIA when tested by double immunodiffusion technique gave a single band. Immunoinhibition studies confirmed the generation of antibodies. These results clearly indicated that GS-IIA was purified to homogeneity. The H2N-terminal amino acid determination enabled us to identify leucine as the amino acid which was a further check of the purified protein. The purified GS-IIB was not immunogenic in property and did not generate antibodies. Double immunodiffusion and immunoinhibition verified the nonimmunogenic characteristic of GS-IIB. Also, the antibodies made against GS-IIA did not cross-react with GS-IIB enzyme form. The H2N-terminal amino acid determination and SDS-PAGE together, however, confirmed the homogeneity of GS-IIB. The purified GS-IIA was investigated in greater details. It showed a sharp pH optima of 7.4, donor-substrate specificity for UDP-glucose. The Km and Vmax values for UDP -glucose substrate were 0.67 mM and 6.25 μmol. min^{-1}. mg^{-1} respectively. The enzyme showed a requirement for Ca^{2+} which was asserted by the fact that EGTA, a chelating agent inhibited the GS-IIA activity. In contrast Zn^{2+} and Mn^{2+} inhibited the enzyme activity *in vitro*. It was also inhibited by nucleotides; ATP, GTP, CTP, UDP,and UMP. However among the 5'- nucleoside monophosphates, only the UDP and UMP inhibited the enzyme

activity by 40-45% whereas inhibitory effect of ADP, GDP, AMP or GMP was only marginal varying from 20% to 11%. It was also observed that dol-P, the carrier lipid of saccharides in the glycosylation of proteins in eukaryotic cells had no significant effect on the activity of the enzyme.

The effect of fluoride ions on GS-IIA was studied and it was observed that at a concentration of 20 mM the stimulation was 1.5 times. The nature of inhibition of ATP, GTP and CTP on GS-IIA was investigated. The inhibition by ATP and CTP was of competitive type and that for GTP was of noncompetitive type.

The study of the properties of the purified GS-IIB showed that the pH optima was 7.0. Infact at pH 7.4, the optima of GS-IIA, the activity of GS-IIB was 60% of the maximum. Like Gs-IIA, the activity of GS-IIB was also inhibited by ATP, GTP, CTP, UDP and UMP, although the extent of inhibition of the latter was relatively smaller than the former. The Lineweaver-Burk plot gave an indication of the Km and Vmax values which were 14.3 mM and 8.3 μmoles. $min^{-1}mg^{-1}$, respectively.

7.0 REFERENCES

1. Addison, R. and Scarborough, G.A. (1981): Solubilization and purification of the Neurospora plasma membrane, H^+-ATPase. J. Biol. Chem. **256**, 13166-13171.

2. Aist, J.R. (1976): Papillae and related wound plugs of plant cells. Annu. Rev. Plant Pathology **14**, 145-163.

3. Anderson, M.A., Hoggart, R.D. and Clarke, A.E. (1983) : The possible role of lectins in mediating plant-cell interactions. Prog. Clin. Biol. Res. **138**, 143-161.

4. Bacon, J.S.D., Davidson, E.D., Jones, D. and Taylor, I.F. (1966): The location of chitin in the yeast cell wall. Biochem. J. **101**, 36 C - 38 C

5. Barber, G.A., Elbein, A.D. and Hassid, W.Z. (1964) : The synthesis of cellulose by enzyme systems from higher plants. J. Biol. Chem. **239**, 4056-4061

6. Bartnicki - Garcia, S. (1968) : Cell wall chemistry, morphogenesis and taxonomy of fungi Annu. Rev. Microbiol. **22**, 87-108

7. Bell, A.A. (1981) : Biochemical mechanisms of disease resistance. Annu. Rev. Plant Physiol. **32**, 21-81.

8. Beran, K., Holan, Z. and Baldrian, J. (1972) : The chitin - glucan complex in Sacharomyces cerevisiae I. IR and X-ray observations. Folia Microbiol. (Prague) **17**, 322-330.

9. Blaschek, W., Semler, U. and Franz, G. (1985): The influence of potential inhibitors on the in vivo and in vitro cell wall β- glucan biosynthesis in tobacco cells. J. Plant Physiol. **120**, 457-470.

10. Bowman, B.J., Blasco, F. and Slayman, S.W. (1981): Purification and characterization of the plasma membrane ATPase of Neurospora crassa. J. Biol. Chem. **256**, 12343-12349.

11. Brett, C.T. (1978) Synthesis of β - (1 -> 3) - glucan from extracellular uridine diphosphate glucose as a wound response in suspension - cultured soybean cells. Plant Physiology, **62**, 377 - 382.

12. Brett, C.T. and Northcote, D.H. (1975) : The formation of oligoglucans linked to lipid during synthesis of β - glucan characterized membrane fractions isolated from peas. Biochem. J. **148**, 107-117.

13. Brown, E.G. and Short, K.C. (1969) : The changing nucleotide pattern of sycamore cells during culture in suspension. Phytochem. **8**, 1365 - 1372.

14. Brummond, D.A. and Gibbons A.P. (1964) : The enzymatic synthesis of cellulose by higher plants. Biochem. Biophys. Res. Commun. **17**, 156-159.

15. Brummond, D.A. and Gibbons, A.P. (1965) : Enzymatic cellulose synthesis from UDP - D - glucose [^{14} C] by Lupinus albus. Biochem. **2, 342**, 308-318.

16. Callaghan, T., Ross, P., Weinberger-Ohana, P., Garden, G. and Benziman, M. (1988) : β-Glucoside activators of mung bean UDP- glucose : β-glucan synthase. 1. Identification of an endogenous β-linked glucolipid activator. Plant Physiol.**86**, 1099-1103.

17. Cheung, W.Y. (1980) : Calmodulin plays a pivotal role in cellular regulation Science. **207**, 19-27.

18. Cumming, D.F. (1970) : Separation and identification of soluble nucleotides in cambial and young xylem tissue of Larix decidua Mill. Biochem. J. **116**, 189-198.

19. Davis, L.G., Dibner, M.D. and Battey, L.F. (1986) : Silver staining for gels. In : Basic methods in molecular biology. Elsevier, New York, pp 315-317.

20. Delmer, D.P., Heiniger, U. and Kulow, C. (1977) : UDP-glucose : glucan synthetase in developing cotton fibers. I. Kinetic and physiological properties. Plant Physiol. **59**,713-718.

21. Delmer, D.P., Benziman, M. and Padan, M. (1982) : Requirement for a membrane potential for cellulose synthesis in intact cells of Acetobactor xylinum. Proc. Natl. Acad. Sci. USA, **79**, 5282-5286.

22. Delmer, D.P., Thelen, M. and Marsden, M.P.F. (1984): Regulatory mechanisms for the synthesis of β-glucans in plants. In: Structure, Function and Biosynthesis of Plant Cell Walls. (Dugger, W.M. and Garcia, S.B., eds.) Rockville, Md, USA: American Society of Plant Physiologists. pp. 133-149.

23. Delmer, D.P. (1987) : Cellulose biosynthesis. Annu. Rev. Plant Physiol.. **38**, 259-290.

24. Delmer, D.P. and Solomon, M. (1989): Identification of polypeptides involved in plant β -glucan synthesis. In : Abstr., 5th Cell Wall Meeting, Edinburgh, Abstr. 36.

25. Dhugga, K.S. and Ray, P.M. (1991) : Isoelectric focussing of plant plasma membrane proteins. Further evidence that a 55 kilodalton polypeptide is associated with β-1,3-glucan synthase activity from pea. Plant Physiol. **95**, 1302-1306

26. Eiberger, L.L., Ventola, C.L. and Wasserman, B.P. (1985) : Solubilization of a digitonin - stable glucan synthase from red beet root Plant Sci. Lett. **37**, 195-198.

27. Eiberger, L.L. and Wasserman, B.P. (1987) : Partial purification of digitonin solubilized β-glucan synthase from red beet root. Plant Physiol. **83**, 982-987.

28. Elnaghy, M.A. and Nordin, P. (1966) : Soluble nucleotides of wheat and oat seedling Arch. Biochem. Biophys. **113**, 72-76.

29. Eschrich, W. (1975): Sealing systems in plants In: Transport in plants. I. Phloem transport, Encyclopaedia of Plant Physiology N.S. Vol. I. (Zimmerman, M.H. and Milburn, J.H. eds.) Springer-Verlag Berlin Heidelberg New York. pp, 39-56.

30. Feingold, D.S., Neufeld, E.F. and Hassid, W.Z. (1958) : Synthesis of a β-(1-3) linked glucan by extracts of Phaseolus aureus seedlings. J. Biol. Chem. **233**, 783-788.

31. Fevre, M. (1983): Nucleotide effects on glucan-synthesis activities of particulate enzymes from Saprolegnia. Planta. **159**, 130-135.

32. Fincher, G.B. and Stone, B.A. (1981) : Metabolism of noncellulosic polyaccharides. In : Plant Carbohydrates II. Encyclopaedia of Plant Physiology, New Series Vol. 13 B. (Tanner, W. and Loewus, F.A., eds.) pp. 68-114.

33. Fink, J., Jeblick, W. and Kauss, H. (1990) : Partial purification and immunological characterization of 1,3-β - glucan synthase from suspension cells of Glycine max. Planta. **181**, 343-348.

34. Fiske, C.H. and SubbaRow, Y. (1925) : The colourimetric determination of phosphorous. J. Biol. Chem. **66**, 375-400.

35. *Fraenkel-Conrat, H. and Harris, J.I. (1954) : N-terminal sequence determination. In : Techniques in protein chemistry (Legget Bailey, J., ed.) 1962, pp. 143-201.

36. Franz, G. (1969) : Soluble nucleotides in developing cotton hair. Phytochemistry. **8**, 737-741.

37. Frost, D.J., Read, S.M., Drake, R.R., Haley, B.E. and Wasserman, B.P. (1990): Identification of the UDP-glucose-binding polypeptide of callose synthase from Beta vulgaris L. by photoaffinity labeling with 5-Azido-UDP-glucose. J. Biol. Chem. **265**, 2162-2167.

38. Gilroy, S., Hughes, W.A. and Trewavas, A.J. (1986) : The measurement of intracellular calcium levels in protoplasts from higher plant cells. FEBS Lett. **199**, 217-221.

39. Girard, V. and Maclachlan, G. (1987) : Modulation of pea membrane β-glucan synthase activity by calcium, polycation, endogenous protease and protease inhibitor, Plant Physiol. **85**, 131-136.

40. Glaser, L. (1958) : The synthesis of cellulose in cell-free extracts of Acetobacter xylinum. J. Biol. Chem. **232**, 627-636.

41. Gorin, P.A.J. and Spencer, J.F.T. (1968) : Structural chemistry of fungal polysaccharides. Adv. Carbohydrate Chem. **23**, 367-417.

42. Gordon, R. and Mclachlan, G. (1989) : Incorporation of UDP-[^{14}C] glucose into xyloglucan pea membranes. Plant Physiol. **91**, 373- 378.

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

44. Griffing, L.R., Mersey, B.G. and Fowke, L.C. (1986) : Cell fractionation analysis of glucan synthase-I and II. Distribution and polysaccharide secretion in soybean protoplasts. Planta. **167**, 175-182.

45. Hanke, B.E. and Northcote, B.H. (1974) : Cell Wall formation by callus protoplasts. J. Cell. Sci. **14**, 29-50.

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

47. Hayashi, T., Read, S.M., Bussell, J., Thelen, M., Lin, F.C., Brown, R.M. Jr. and Delmer, D.P. (1987). UDP-glucose (1-3) - β - glucan synthases from mung bean and cotton: Differential effects of Ca^{2+} and Mg^{2+} on enzyme properties and on macromolecular structure of the glucan product. Plant Physiol. **83**, 1054-1062.

48. Hayashi, T. and Matsuda, K. (1981) : Biosynthesis of xyloglucan in suspension cultured soybean cells. Evidence that the enzyme system of xyloglucan synthesis does not contain 1,4-β-D-glucan 4-β-D-glucosyl transferase activity (EC 2.4.1.12). Plant Cell Physiol. **22**, 1571-1584.

49. Heiniger, U. (1983): UDP-glucose : 1,3-β-glucan synthase in potato tubers; solubilization and activation by lipids. Plant Science Lett. **32**, 35-41.

50. Heiniger, U. and Delmer, D.P. (1977) : UDP-Glucose : Glucan synthetase in developing cotton fibre II. Structure of the reaction product. Plant Physiol. **59**, 719-723.

51. Henry, R.J. and Stone, B.A. (1983): Solubilization of β-glucan synthases from the membranes of cultured ryegrass endosperm cells. Biochem. J. **203**, 629-636.

52. Henry, R.J., Schibeci, A. and Stone, B.A. (1983) : Localization of β-glucan synthetases on the membranes of cultured Lolium multiflorum(ryegrass) endosperm cells. Biochem. J. **209**, 627-633.

53. Houwink, A.L. and Kreger, D.R. (1953) : Observations on the cell wall of yeasts. An electron microscope and X-ray diffraction study. Antonie Vaan Leeuwenhoek J. Microbiol. Serol. **19**, 1-24.

54. Jacob, S.R. and Northcote, D.H. (1985): In vitro glucan synthesis by membranes of celery petioles : The role of the membrane in determining the type of linkage formed. J. Cell Sci. Suppl. **2**, 1-11.

55. Jaffe, M.J., Biro, R., and Bridle, K. (1983): Thigmomorphogenesis: Calibration of the parameters of the sensory function in beans. Plant Physiol. **49**, 410-416.

56. Jaffe, M.J. and Leopold, A.C. (1984) : Callose deposition during gravitropism of Zea mays and Pisum sativum and its inhibition by 2-deoxy-D-glucose. Planta. **161**, 20-26.

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

58. Kauss, H. (1985) : Callose biosynthesis as a Ca^{2+}-regulated process and possible relations to the induction of other metabolic changes. J. Cell Science Suppl. **2**, 89-103.

59. Kauss, H. (1986) : Ca^{2+} - dependence of callose synthesis and the role of polyamines in the activation of 1,3-β-glucan synthase by Ca^{2+}. In : Molecular and Cellular Aspects of Calcium in Plant Development. (Trewavas, A.J. ed.) Plenum Publishing Corporation. pp 131-137.

60. Kauss, H. (1987): Some aspects of calcium dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. **38**, 47-72.

61. Kauss, H. and Jeblick, W. (1985) : Activation by polyamines, polycations and ruthenium red of the Ca^{2+} - dependent glucan synthase from soybean cells. FEBS. Lett. **185**, 226-230.

62. Kauss, H. and Jeblick, W. (1986) ; Synergistic activation of 1,3-β-D-glucan synthase by Ca^{2+} and polyamines. Plant Science. **43**, 103-107.

63. Kauss, H., Kohle, H. and Jeblick, W. (1983) : Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from

soybean cells. FEBS Lett. **158**, 84-88.

64. Kemp, J. and Loughman, B.C. (1973) : Chain initiation in glucan biosynthesis in mung beans. Biochem Soc. Trans. **1**, 446-448.

65. Kemp, J. and Loughman, B.C. (1974) : Cyclitol glucosides and their role in the synthesis of a glucan from uridine diphosphate glucose in Phaseolus aureus. Biochem. J. **142**, 153-159.

66. Kemp. J., Loughman, B.C., Ephritikhine, G. (1978) : The role of inositol glucosides in the synthesis of β-1,3 glucans. In : Cyclitoles and Phosphoinositides. (Wells, W.W. and Eisenberg, F. Jr, eds.) Academic Press, New York. pp 439-449.

67. Klein, A. S., Montezinas, D. and Delmer, D.P. (1981) : Cellulose and β(1-3) glucan synthesis during the early stages of wall regeneration in soybean protoplasts. Planta. **152**, 105-114.

68. Kohle, H., Jeblick, W., Poten, F., Blaschek, W. and Kauss, H. (1985) : Chitosan - elicited callose synthesis in soybean cells as a Ca^{2+} - dependent process. Plant Physiol. **77**, 544-551.

69. Kornfeld, R. and Kornfeld, S. (1985) : Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54**, 631-664.

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

71. Langebartels, C., Seitz, V. and Seitz, H.V. (1981) : β-glucan synthetase activities in regenerating protoplasts from carrot suspension cultures. Plant Sci. Lett. **22**, 327-335.

72. Larriba, G., Morales, M. and Ruiz-Herrera, J. (1981):Biosynthesis of β-glucan microfibrils by cell-free extracts from Saccharomyces cerevisiae. J. Gen. Microbiol. **124**, 375-383.

73. Lawson, S.G., Mason, T.L., Sabin, R.D., Sloan, M.E., Drake, R.R., Haley, B.E. and Wasserman, B.P. (1989) : UDP-glucose : (1,3)-β-glucan synthase from Daucus carota L. Plant Physiol. **90**, 101-108.

74. Leloir, L.F. (1951) : The enzymatic transformation of uridine diphosphate glucose into a galactose derivative. Arch. Biochem. Biophys. **33**, 186-190.

75. Leloir, L.F. (1964) : Nucleoside diphosphate sugars and saccharide synthesis. Biochem. J. **91**, 1-8.

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

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

78. Mandala, S. and Taiz, L. (1985): Partial purification of the tonoplast ATPase from corn coleoptiles. Plant Physiol. **78**, 327-333.

79. Mason, T.L. and Wasserman, B.P. (1987) : Inactivation of red beet β-glucan synthase by native and oxidised phenolic compounds. Phytochem. **26**, 2197-2202

80. Mayer, R.J. and Walker, J.H. (1987) : Technical Supplement; Antiserum Processing. In : Immunochemical Methods in Cell and Molecular Biology. Academic Press, pp. 193-284.

81. McNairn, R.B. (1972): Phloem translocation and heat induced callose formation in field grown Gossypium hirsutum L. Plant Physiol. **50**, 366-370.

82. Morrow, D.L. and Lucas, W.J. (1986) : (1-3)-β-D-glucan synthase from sugar beet. Plant Physiol. **81**, 171-176.

83. Northcote, D.H. (1985): Control of cell wall formation during growth In: Biochemistry of plant cell walls. Society for Experimental Biology Seminar series. 28. (Brett, C.T. and Hillman, J.R., eds.) pp. 177-197.

84. Olaisson, H., Mardh, S. and Arvidson, G. (1985): Phospholipid organization in H, K-ATPase - containing membranes from pig gastric mucosa. J. Biol. Chem. **260**, 11262-11267.

85. Ordin, L. and Hall, M.A. (1967) : Studies of cellulose synthesis by a cell-free oat coleoptile enzyme system : Inactivation by airborne oxidants. Plant Physiol. **42**, 205-212.

86. Orlean, P.A.B. (1982) : (1-3)-β-D-glucan synthase from budding and filamentous cultures of the dimorphic fungus Candida albicans. Eur. J. Biochem. **127**, 397-403.

87. Orlean, P.A.B. and Ward, S.M. (1983) : Sodium fluoride stimulates (1,3)-β-D-glucan synthase from Candida albicans. FEMS Microbiol. Lett. **18**, 31-35.

88. Ouchterlony, O. and Nilsson, L.A. (1986) : Immunodiffusion and immunoelectrophoresis In : Handbook of experimental immunology. (Weier, D.M. eds.), Vol. I, pp 32.1-32.50, Immunochemistry, Blackwell Scientific Publications.

89. Paliyath, G. and Poovaiah, B.W. (1988) : Promotion of β-

glucan synthase activity in corn microsomal membranes by calcium and protein phosphorylation Plant cell Physiol. **29(1)**, 67-73.

90. Percival, E. and McDowell, R.H. (1981) : Algal walls - composition and biosynthesis. In : Plant Carbohydrates II. Extracellular Carbohydrates. (Tanner, W. and Loewus, F.A. eds.) pp 279-316.

91. Peaud-Leonel, C. and Axelos, M. (1970) : Structural features of the β-glucans enzymatically synthesized from UDP-glucose by wheat seedling. FEBS Lett. **8**, 224-228.

92. Ray, P.M. (1977) : Auxin-binding sites of maize coleoptiles are localized on membranes of the endoplasmic reticulum. Plant Physiol. **59**, 434-438.

93. Raymond, Y., Fincher, G.B. and Maclachlan, G.A. (1978) : Tissue slice and particulate β-glucan synthetase activities from Pisum epicotyls. Plant Physiol. **61**, 938-942.

94. Read, S.M. and Delmer, D.P. (1987) : Inhibition of mung bean UDP-glucose : (1-3)β glucan synthase by UDP - pyridoxal. Evidence for an active-site amino group. Plant Physiol. **85**, 1008-1015.

95. Riehl, T.E. and Jaffe, M.J. (1984) : Physiological studies on pea tendrils. XIV. Effects of mechanical perturbation, light and 2- deoxy-D-glucose on callose deposition and tendril coiling. Plant Physiol. **75**, 679-687.

96. Robinson, D.G., Andrea, M., Depta, H., Hartmannn, D. and Hillmer, S. (1987) : Coated vesicles in plants : characterization and function. In: Plant Membranes. Structure. Function. Biogenesis. UCLA Symp. Mol. Cell. Biol. (NS) **63**, 341-358.

97. Robinson, D.G. and Depta, H. (1988) : Coated vesicles. Annu. Rev. Plant Physiol. **39**, 53-99.

98. Rosenberger, R.F. (1976) : The cell wall In: The filamentous fungi. Vol. II. Biosynthesis and metabolism (Smith, J.E. and Berry, D.R. eds), pp. 328-344.

99. Sharma, C.B., Lehle, L. and Tanner, W. (1982) : Solubilization and characterization of the initial enzymes of dolichol pathway from yeast. Eur. J. Biochem. **126**, 319-325.

100. Sharma, C.B., Mittal, R. and Tanner, W. (1986) : Purification and properties of a glycoprotein adenosine - monophosphatase from the plasma membrane fraction of Arachis hypogaea cotyledons. Biochem. Biophys. Acta. **480**,

417-427.

101. Shematek, E.M. and Cabib, E. (1980): Biosynthesis of the yeast cell wall. II. Regulation of β-(1-3)-glucan synthetase by ATP and GTP. J. Biol. Chem. **255**, 895-902.

102. Shematek, E.M., Braatz, J.A. & Cabib, E. (1980): Biosynthesis of the yeast cell wall. I. Preparation and properties of β(1-3) glucan synthetase. J. Biol. Chem. **255**, 888-894.

103. Shimomura, T. and Dukstra, J. (1975) : The occurence of callose during the process of local lesion formation. Neth. J. Plant Pathology. **81**, 107-121.

104. Shore, G., Raymond, Y. and Maclachlan, G.A. (1975): Cell-surface and intracellular cellulose synthetase activities in relation to the stage and direction of cell groth. Plant Physiol. **34-38**.

105. Sloan, M.E., Rodis, P. and Wasserman, B.P. (1987) : CHAPS solubilization and functional reconstitution of β-glucan synthase from red beet (Beta vulgaris L.) storage tissue Plant Physiol. **85**, 516-522.

106. Sloan, M.E. and Wasserman, B.P. (1989) : Susceptibility of UDP-glucose : (1,3)-β-glucan synthase to inactivation by phospholipases and trypsin. Plant Physiol. **89**, 1341-1344.

107. Smith, M.M. and Stone, B.A. (1973) : β-glucan synthesis by cell-free extracts from Lolium multiflorum endosperm. Biochim. Biophys. Acta. **313**, 72-94.

108. Smith, R. and Scarborough, G.A. (1984): Large-scale purification of the Neurospora plasma membrane H^+ ATPase. Anal. Biochem. **138**, 156-163.

109. Solms, J. and Hassid, W. Z. (1957) : Isolation of uridine diphosphate N-acetyl-glucosamine and uridine diphosphate glucuronic acid from mung bean seedlings. J. Biol. Chem. **228**, 357-364.

110. Stoddart, R.W. (1984) : Polysaccharide assembly in eucaryotes. In : The Biosynthesis of Polysaccharides (Croom Helm, London and Sydney) pp. 179-260.

111. **Su, J.S. (1965) : Carbohydrate metabolism in the shoots of bamboo. III Separation and identification of nucleotides. J. Chin. Agri. Chem. Soc., pp 45-54.

112. Tacke, M., Yang, Y. and Stemp, M. (1991): Multiplicity of soluble glucan - synthase activity in spinach leaves - Enzyme pattern and intracellular location. Planta. **185**, 220-226.

113. Trevelyan, W.E., Procter, D.P. and Harrison, J.S. (1950): Detection of sugars on paper chromatograms. Nature **166**, 444-445.

114. Tsai, C.M. and Hassid, W.A. (1971): Solubilization and separation of uridine diphospho-D-glucose: β-(1,4)-glucan and uridine diphospho-D-glucose: β-(1,3)-glucan glucosyl transferases from coleoptiles of Avena sativa. Plant Physiol. **47**, 740-744.

115. Tsai, C.M. and Hassid, W.Z (1973): Substrate activation of β - (1-3) - glucan synthetase and its effect on the structure of a glucan obtained from UDP - D - glucose and particulate enzyme of oat coleoptiles. Plant Physiol . **51**, 998 - 1001.

116. Van der Woude, W.J., Lembi, C.A., Morre, D.J., Kindinger, J.I. and Ordin, L. (1974) : β - glucan synthetase of plasma membrane and Golgi apparatus from onion stems. Plant Physiol. **54**, 333-340.

117. Vithanage, H.I.M.V. and Knox, R.B. (1977): Development and cytochemistry of stigma surface and response to self and foreign pollination in Helianthus annuus. Phytomorphology. **27**, 168-179.

118. Waldmann, T., Jeblick, W. and Kauss, H. (1988) : Induced net Ca^{2+} uptake and callose biosynthesis in suspension cultured plant cells. Planta. **173**, 88-95.

119. Wasserman, B.P. and McCarthy, K.J. (1986) : Regulation of plasma membrane β-glucan synthase from red beet root by phospholipids. Reactivation of Triton X-100 extracted glucan synthase by phospholipids. Plant Physiol. **82**, 396-400.

120. Williamson, R.E. (1981) : Free Ca^{2+} concentration in the cytoplasm : A regulator of plant cell function, What's new ? Plant Physiol. **12**, 45-48.

* Cross reference.

Cross reference obtained from Encyclopedia of Plant Physiology, New Series **Vol. 13B. (Tanner, W. and Loewus, F.A., eds).