# STUDIES ON MULTIMOLECULAR FORMS OF ALPHA GALACTOSIDASE OF CICER ARIETINUM SEEDS

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By

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Dedicated

to

my Grand father

(Late Shri J.N. Gupta)

and

Beloved children

Shalini & Saurabh

## CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **STUDIES ON MULTIMOLECULAR FORMS OF ALPHA GALACTOSIDASE OF CICER ARIETINUM SEEDS** in fulfilment of the requirment for the award of the Degree of Doctor of Philosophy submitted in the Department of **BIO-SCIENCES AND BIO-TECHNOLOGY** of the University is an authentic record of my own work carried out during a period from **February 1982** to **January 1988** under the supervision of **Dr. C.B. SHARMA, Professor & Head, Department of Bio-Sciences 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.

Candidate's Signature

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

Date Jan. 22, 1988.

C. B. Sharma

Signature(s) of Supervisor's

The candidate has passed the viva-voce examination held on ----------

at _____. The thesis is recommended for award of the Ph.D. Degree.

Signature of Guide/s

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STUDIES ON MULTIMOLECULAR FORMS OF ALPHA GALACTOSIDASE OF *CICER ARIETINUM* SEEDS

## ABSTRACT

Three molecular forms of α-galactosidase, one high molecular weight form (α-galactosidase I) and two low molecular weight forms (α-galactosidase IIa and α-galactosidase IIb) have been separated from the 6 days germinated cotyledons of *Cicer-arietinum* seeds using acetone treatment, pH precipitation, ammonium sulphate fractionation, gel filtration and ion exchange chromatography. The low molecular weight forms (α-galactosidase IIa and α-galactosidase IIb) showed a single protein band on polyacrylamide gel electrophoresis with electrophoretic mobilities, relative to bromophenol blue, of 0.24 and 0.32, respectively. The apparent molecular weights of α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb, as determined by Sephadex gel filtration method, were found to be 149,600, 56,000 and 50,000 daltons, respectively. On SDS polyacrylamide gel electrophoresis, under completely reduced conditions, α-galactosidase IIa yielded three distinct protein bands corresponding to molecular weight 36,000, 22,000 and 16,000 daltons and α-galactosidase IIb yielded two bands of molecular weight 40,000 and 18,000 daltons, respectively, indicating that both enzymes have subunit structure.

The high and low molecular forms of α-galactosidase showed identical pH profiles. The substrate specificities in principle, were same with difference in quantitative values. The pattern of enzyme inhibition by metal ions and sugar analogus was similar, however, the degree of inhibition was significantly different for each form of the enzyme.

The $K_m$ values of α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb were found to be 0.36, 0.14 and 0.28 mM and $V_{max}$ values were 5.3, 2.0 and 1.0 μmol/min/mg, respectively. The various α-galactosidase forms showed markedly different thermodynamic parameters. The purified α-galactosidase IIa, α-galactosidase IIb and the reconstituted high molecular weight α-galactosidase are glycoproteins containing 9.4%, 29.5% and 25% carbohydrate, respectively, composed of N-acetylglucosamine and mannose.

The immobilized isoenzymes in calcium alginate beads exhibited higher pH tolerance and greater thermal stability as compared to the free enzyme. The immobilized enzyme did not follow the michaelis-Menten kinetics.

Developmental studies of α-galactosidase isoenzymes during germination and maturation of seeds showed that the number of molecular forms of α-galactosidase and their relative concentrations varied with physiological state of the seed. In green immature seeds (10 days after flowering), the total activity was very low, with only low molecular weight form (α-galactosidase II). After 17 days of flowering high molecular weight

form (α-galactosidase I) made its appearance and a marked increase in total activity of α-galactosidase occurred. From 25th to 40 days after flowering, the level of high molecular weight form increased with a simultaneous decline in the level of α-galactosidase II. During germination relative level of α-galactosidase I activity which was predominant in dormant seeds continuously declined with a concomitant increase of α-galactosidase II level and finally on seventhday of germination when the metabolic activities were at peak the lower molecular weight α-galactosidase was highly predominant with only very small proportion of α-galactosidase I. Thus, activity ratio of α-galactosidase I to α-galactosidase II was completely reversed during the process of germination.

The conversion of low molecular weight forms into high molecular weight form α-galactosidase in vitro on storage shows that these forms are derived from each other depending on the physiological state of the seed.

Treatment with cycloheximide during germination inhibited α-galactosidase synthesis by 41% and has reversed the pattern of evolution of multimolecular forms of α-galactosidase, which appeared to assume the isoenzyme pattern of the maturing seeds

The different varieties of Cicer-arietinum seeds with clearly distinguishable superfacial features have also been found to contain different isoenzymic patterns of α-galactosid

isoenzymes. For instance, in all white varieties of chickpea seeds the high molecular weight form of α-galactosidase was lacking. Instead, a very low molecular weight form (Mr=25,000 daltons) was found in these varieties which was not present in any of the black variety seeds. On the other hand all the black varieties of chickpea were characterized by the presence of high molecular weight form α-galactosidase (Mr=149,600 daltons). The number of multimolecular forms and their relative intensity vary from variety to variety and is a function of the physiological state of the seed.

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(Mrs. YASHODA MITTAL)

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# 1.0 INTRODUCTION

The physiological and developmental roles of isoenzymes have been the subject of comparatively few studies, although isoenzymic patterns are specific to the genetic variety and developmental stage of the plant. The stage of development at which the isoenzymes appear or disappear relates primarily to the 'turning on' or derepression of genes controlling or modifying the synthesis of the enzyme (190). Sometimes the factors affecting the plant metabolism like mineral absorption, salt stress, disease resistance, cold-hardiness, etc. are known to influence the intensity of isoenzymes activity which may lead to changes in the isoenzymic patterns (125,81). Thus, induction and derepression of isoenzymes under stress environment also provide an early indication of potential adaptation of plants. The isoenzymic pattern of different varieties of a particular seed or different species of the same family of seeds are also indicative of the evolutionary relationship.

Study of isoenzymes, while not without limitations, provides the potential tool for studying some of the important facets concering growth and differentiation, drouth and disease resistance, and analysis of natural population (133, 165). In fact, in several cases the differing patterns of isoenzymes have been used as biological markers in the analysi of natural population as the degree of outbreeding or inbreeding in natural populations can be assessed zymographically (4,25). For example, the occurrence of multiple molecular

forms and their maintenance in natural populations had led to speculations regarding homeostasis and variation in a collection of barley cultivars (4,117) and evaluated the mating system in maize population using isoenzyme polymorphism (25). In maize genetic variation contributed by the seven known peroxidase loci alone can distinguish over a thousand different homozygous inbred lines. Similarily varietal identification of highly self-pollinated crops - e.g., beans, peas, soybeans, tomatoes, etc., could be aided by zymogram analysis (48). Furthermore, one of major advantages of isozyme techniques is that a large variety of tissues can be assayed since tissues like cotyledons, mesocotyle, radicle, etc., provide rich fingerprints in many plants (21,22,85,152).

Multiple forms of α-galactosidase are known to occure in a number of dormant seeds (34,51,80,119) including Cicer arietinum. It is,therefore, resonable to expect that α-galactosidase isoenzymes would be useful in providing a suitable biomarker for the identification of different varieties of Cicer arietinum. Chickpea, popularily known as gram seeds, represent one of the important economic crops of India and is extensively used both by human beings and animals as a major food supplements. A large number of gram varieties are found some of which are distinguishable by their superficial characters such as colour, shape and size, while others can not be so distinguished.To the best of our knowledge no biochemical parameters are available to distinguish one chickpea variety from the other. Previous studies

have shown that chickpea seeds contain multiple forms of α-galactosidase (164). However, purification and characterization of the multiple forms of enzyme was not done. As a result their usefulness in the identification of different varieties, some of which show widely differing response to moisture, temperature, drouth and pest resistance, was not realized.

In the present study we have undertaken the purification and characterization of the major multiple forms of α-galactosidase of chickpeas with a view to use them as genetic/biochemical markers in defining different varieties of chickpeas. The main objectives of the research described in the thesis were as follows:-

1. Isolation, purification and characterization of multimolecular forms of α-galactosidase from chickpea cotyledons.

2. To study the developmental pattern of α-galactosidase isoenzymes during seed germination and maturation.

3. To study the inter-relationship of high molecular weight form and low molecular weight form α-galactosidase, in vitro.

4. To compare the isoenzymic pattern of α-galactosidase isoenzymes in different varieties of Cicer-arietinum seeds.

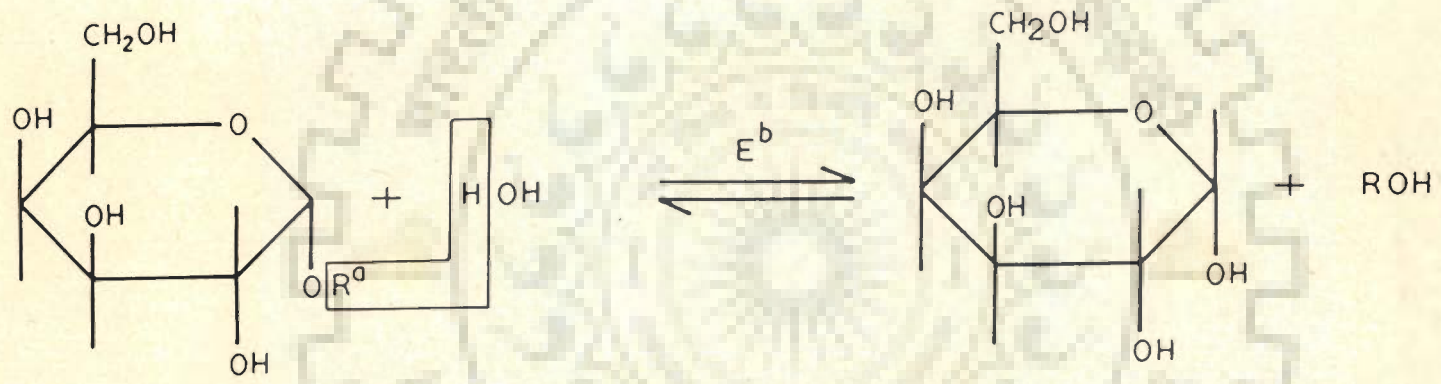
## 2.0 LITERATURE REVIEW

### 2.1 INTRODUCTION

The glycosidic linkages allow the formation of glycans of varying complexity including those present in glycolipids and glycoproteins. These biopolymers are hydrolyzed into monosaccharides by specific glycosidases which occur widely in nature. These enzymes have been extensively studied and purified from a variety of sources and a large body of data is available on the specificity, mechanism of action and physiological role. The importance of glycosidases in the metabolism of carbohydrates in plants, particularly in storage organs such as seeds, is well recognized. Recently, an excellent review on plant glycosidases has been published by Dey and Elena del Campillo (62). The present literature survey is limited to plant α-galactosidases only.

### 2.2 OCCURRENCE

α-galactosidase (α-D-galactoside-galactohydrolase, E.C. 3.2.1.22 ;33) catalyzes the hydrolysis of glycosides in which α-galactosyl residue forms the nonreducing terminal (Fig 1). This enzyme is widely distributed in plant Kingdom (49,135,164,182,191) and its presence can be predicted in all plants (and their organs) that contain α-D-galactosyl-containing oligosaccharides. In fact, galactosyl oligosaccharides rank next to sucrose in their abundance in the plant Kingdom (57) and comprehensive lists of plant sources containing α-galactosidase activity are available (61, 62). Some important plant sources of α-galactosidase are summerized in Table I. In addition, this




[a]R _ Alkyl, Aryl, Monoglycosyl or Polyglycosyl gp.

[b]E _ Alpha galactosidase

Fig.1. Reaction Catalyzed By ∝-Galactosidases.

TABLE I SOME IMPORTANT PLANT SOURCES OF α-GALACTOSIDASES

| Organism | Reference | Organism | Reference |
|---|---|---|---|
| Acer pseudoplatanus | 11 | Glycine max | 119 |
| Aerobacter aerogenes | 86 | Laburnum alpinum | 80 |
| Artocarpus lakoocha | 32 | Lespedeza bicolour | 80 |
| Aspergillus spp. | 1,9,168 | Lupinus arboreus | 79 |
| Bacillus stearothermophillus | 45,47,74,152 | Medicago sativa | 90,119,141 |
| Bacteriodes fragilis | 14 | Mortierella vinacea | 175 |
| Bifidobacterium breve | 148 | Phaseolus limensis | 79 |
| Cajanus indicus | 11,52 | Phaseolus vulgaris | 2 |
| Calvatia cyantiformis | 108 | Pisum sativum | 128 |
| Caragana arborescence | 80 | Poterioochromonas malhamensis | 58 |
| Ceratonia siliqua | 119,159 | Sacchromyces carlsbergensis | 108 |
| Cicer arietinum | 164 | Spartium junceus | 80 |
| Cucurbita pepo | 181,182 | Streptococcus bovis | 10 |
| Cyamopsis tetagonolobus | 119,122 | Trigonella foenum graecum | 124,140,141 |
| Cytisus multiflorus | 80 | Trifolium incarnatum | 141 |
| Diplococcus pneumoniae | 107 | Ulex europaeus | 80 |
| Escherichia coli | 26,111,155 | Vicia faba | 139 |
| Genista monosperma | 80 | Vicia sativa | 135 |
| | | Vigna radiata | 77 |

enzyme was found in maize (179), coconut (118), sugarcane (50,51), soybean (82), figs (110), cultivated and wild peas (116), in extracts of wheat flour (189) and in mung beans (130).

## 2.5 LOCALIZATION AND INTRACELLULAR DISTRIBUTION

A survey of the organs of the rat, using a histochemical method, has revealed highest activities in the cytoplasm of epithelial cells of Brunner's glands in the intestine. Besides, the distal segments of the proximal tubes of the kidney, and the thyroid and parathyroid glands also possess α-galactosidase activity (51). This enzyme has also been reported from hen oviduct (7), rat brain (102), chinese hamster kidney (27) Leukocytes of pig and mule (161), from human placenta(195), plasma (18,19,75), spleen (12), Liver (37,38,39,40,156) fibroblasts (3, 69, 84, 136, 188) WBC (63), polymorphonuclear leukocytes (94,144) and from hair roots (66). It has been detected in urine of normal human and from patients of mucolipidosis II and III (100). In plants it was detected in leaves (16,67,169), roots (13,65,179), stems, fruits, nectar, tubers, and particularly in seeds (56,93). In seeds, the enzyme has been detected in cotyledon, endosperm and embryo (142,119). For example, in mung bean seeds the enzyme was present in cotyledons, hypocotyle and plumules (130), while in cucurbita (182,185) it exists throughout the plant.

The α-galactosidase activity is generally located in the soluble fraction of the cytoplasm(cytosol). In several cases, however, the enzyme activity was cell-wall bound (137,184). In Cucurbita leaves (184), a considerable amount of the enzyme is located in the exocellular region, and is loosely associated either with the outer

surface of plasmalemma or in the free space including the cell wall. Most of the enzyme was released during homogenization. However, about 20% remained attached to the particulate material. This activity could be recovered only by cellulase treatment, implying that the activity was occluded within the wall matrix during the secondary wall deposition. Significant activities of the enzyme has also been detected in the chloroplasts (72) and protein bodies from seeds (77).

## 2.4 ENZYME ASSAY

The reducing disaccharide melibiose, or the nonreducing trisaccharide raffinose, are the natural substrates of the plant enzyme and are often used for the enzyme assay. The liberated hexose molecules can be measured by monitoring the increase in reducing power. Alternatively, the liberated D-galactose can be measured enzymatically using D-galactose dehydrogenase (58).

Synthetic substrates, such as phenyl-, ortho-, or para-substituted nitrophenyl α-D-galactosides are commonly used for routine enzyme assays. The liberated nitrophenols are estimated using absorbance of the yellow colour at 420 nm under alkaline conditions. Methylumbelliferyl α-D-galactoside was used as the substrate for the fluorimetric methods of α-galactosidase assays (58,42,92).

The enzyme is located on gels by 1-naphthyl α-D-galactoside or 6-bromo-2 naphthyl α-D-galactoside (15). The liberated water-insoluble aglycones are coupled with Fast Blue BB, resulting in the formation of a coloured zone which indicates the position of enzyme on gels.

## 2.5 SPECIFICITY OF INDUCTION OF α-GALACTOSIDASE

Bacterial α-galactosidases are not constitutive in nature. They could be induced by several α-galactosides. A number of sugars and derivatives have been tested for their ability to induce the synthesis of α-galactosidase from Saccharomyces carlsbergensis. Beside galactose and the substrates of the enzyme melibiose, raffinose and stachyose, D-galacturonic acid, L-arabinose, D-tagatose, methyl-α-D-galactoside, lactose and isopropyl β-D-thiogalactoside are reported to act as inducers (89). In Bacillus stearothermophilu α-galactosidases were synthesized constitutively when strain AT-7 was grown on tryptone yeast extract medium (152). It is evident that only molecules which contain a galactosidic residue or those which are structurally very similar to this hexose can induce the synthesis of α-galactosidase, the exception being p-nitrophenyl-α-D-galactoside. The lack of induction by this compound might be due to the lethal effect of p-nitrophenol released during its hydrolysis, since the compound is hydrolyzed by the enzyme as efficiently as melibiose (105). Fumigation of 3-4 years old beeches (Fagus sytvatica)with 0.05 - 0.2 ppm $SO_2$ for 4 weeks stimulated α-galactosidase (24). Aspergillus awamori NRRL 4869 cultured on the solid substrate wheat bran produced α-galactosidase (168).

## 2.6 MULTIPLE FORMS OF α-GALACTOSIDASE

The first report of the existence of two molecular weight forms of α-galactosidase was from Vicia faba (49,50). A number of sources are now known to contain multiple forms of the enzyme which have been separated on the basis of well-defined protein character-

istics (Table II). For instance, Barham, et al.(11) isolated multimolecular forms of α-galactosidase from various dormant seeds using Sephadex gel filtration. The presence of the multiple forms of α-galactosidase have also been demonstrated in human leukocytes (15,97), skin fibroblasts (15), liver (97) and placenta (194), using electrofocusing and electrophoresis techniques.

## 2.7 ROLE

The primary role of α-galactosidases in the storage organs of plants is to mobilise the reserve α-D-galactosyl containing olgo-and polysaccharides (11,51,139). In leguminous seeds such as fenugreek , guar, locust and honey locust, where cotyledons and endosperm coexist at maturity, the carbohydrate reserve in the endosperm is not starch but galactomannan (124). In fenugree this polysaccharide is found as an inner, thick deposit on the endosperm wall (124) and, in addition to its role as a carbohydrate reserve, may function through its waterbinding capacity to protect the germinating seed from desication (143). During germination hydrolases secreted from aleurones are actively involved in the degradation of galactomannan to be used as a readily available energy source during germination and early period of plant growth (122,124,125,140,141,142). The hydrolase involved in galactomannan depolymerization include (1 → 4) β-mannan hydrolase, β-mannosidase and α-galactosidase (119,120, 142,159). However, so far there is no report showing direct correlation between α-galactosidase activity and growth (129).

TABLE II SOURCES OF MOLECULAR FORMS, THEIR SEPARATION, PURIFICATION AND PROPERTIES OF SOME PLANT α-GALACTOSIDASES

| Source | Form | Method of separation | Molecular weight | pH optimum | km (mM) | Reference |
|---|---|---|---|---|---|---|
| Caragma arborescence (seed) | I | Gel filtration | 135,000 | 5.0 | 0.80 | 80 |
| | II | | 34,000 | 4.5 | | |
| Castanea sativa (seed) | I | Gel filtration | 215,000 | 4.5 | - | 57 |
| | II | | 55,000 | 6.0 | - | |
| Ceratonia siliqua (seed) | I | DEAE-cellulose | 57,000 | ~5.0 | 0.42 | 119 |
| | II | | 37,000 | ~5.0 | 0.42 | |
| | III | | 25,000 | ~5.0 | 0.45 | |
| Cicer arietinum (seed) | I | Gel filtration | 134,000 | 5.2-6.0 | 0.25 | 164 |
| | II | | 45,000 | 5.0-5.2 | 0.34 | |
| Cucurbita pepo (leaf) | I | DEAE-sephadex | - | 5.7 | 0.37 | 182 |
| | II | | - | 4.6 | 0.57 | |
| | III | | - | 5.6 | 0.04 | |
| | IV | | - | 7.5 | 0.60 | |
| Cyamopsis tetragonolobus (seed) | I | DEAE cellulose | 34,000 | ~5.0 | 0.42 | 119 |
| | II | | 24,000 | ~5.0 | 0.63 | |
| Cytisus multiflorus (seed) | I | Gel filtration | 190,000 | - | 1.0 | 80 |
| | II | | 40,000 | - | | |
| Genista monosperma (seed) | I | Gel filtration | 160,000 | - | 0.58 | 80 |
| | II | | 37,000 | - | | |

Contd ....

Table II (Contd.)

| Source | Form | Method of separation | Molecular weight | pH optimum | km (mM) | Reference |
|---|---|---|---|---|---|---|
| Glycine max (seed) | I | DEAE cellulose | 130,000-150,000 | ~5.0 | 0.50 | |
| | II | | 40,000 | ~5.0 | 0.50 | 119 |
| | III | | 40,000 | ~5.0 | 0.50 | |
| | IV | | 25,000 | ~5.0 | 0.57 | |
| Glycine max (seed) | I | Gel filtration | 150,000 | 5.0 | 0.57 | 82 |
| | II | | 40,000 | - | - | |
| Laburnum alp-inum (seed) | I | Gel filtration | 150,000 | - | 1.43 | 80 |
| | II | | 30,000 | | | |
| Lens culinaris (seed) | I | Gel filtration and SDS electro-phoresis | 160,000 | 6.1 | 0.4 | 61 |
| | II | | 40,000 | 4.7 | - | |
| Lespedeza bicolor (seed) | I | Gel filtration | 120,000 | - | 0.47 | 61 |
| | II | | 31,000 | - | | |
| Lupinus arboreus (seed) | I | Gel filtration | 190,000 | - | 0.39 | 80 |
| | II | | 50,000 | - | - | |
| Medicago sativa (seed) | I | DEAE cellulose | 34,000 | ~5.0 | 0.49 | 119 |
| | II | | 23,000 | ~5.0 | 2.36 | |

Contd.....

Table II (Contd.)

| Source | Form | Method of separation | Molecular weight | pH optimum | Km (mM) | Reference |
|---|---|---|---|---|---|---|
| Phaseolus limensis (Seed) | I | Gel filtration | 180,000 | - | 0.38 | 79 |
| | II | | 39,000 | - | - | |
| Spartium junceum (seed) | I | Gel filtration | 160,000 | - | 1.66 | 80 |
| | II | | 30,000 | - | | |
| Trifolium repens (seed) | I | Hydroxylapatite | 41,000 | 3.8 - 4.2 | 6.6 | 195 |
| | II | | 41,000 | 3.6 - 4.4 | 7.0 | |
| | III | | 41,000 | 4.2 - 4.8 | 2.7 | |
| | IV | | 41,000 | 4.2 - 4.6 | 11.1 | |
| Ulex europaeus (seed) | I | Gel filtration | 160,000 | - | 0.67 | 80 |
| | II | | 42,000 | - | | |
| Vicia faba (seed) | I | Gel filtration and SDS electrophoresis | 160,000 | 2.0 and 5.5 | 0.44 | 139,60 |
| | $II^1$ | | 43,000 | 3.0 and 5.2 | 0.97 | |
| | $II^2$ | | 41,000 | 2.0 and 5.5 | 0.33 | |
| Vigna radiata (seed) | I | Gel filtration | 160,000 | 5.6 and 7.2 | 0.2 | 77 |
| | II | | 40,000 | 5.6 | 0.1 | |

A low level of these enzymes exists in the early stage of germination. At later stages, increase in the enzyme level coincided with galactomannan degradation in the endosperm. The embryo had a relatively high level of the enzyme, which remained constant during germination, and was responsible for the hydrolysis of raffinose family of sugar in this organ. However, for some unknown reason α-galactosidases have great difficulty in cleaving the terminal α-galactosyl linkage present in sphingoglycolipids and glycoproteins. For instance, crystalline α-galactosidase from Mortierella vinacea cleaves melibiose and raffinose efficientl but can not hydrolyze the α-galactosyl residues present in glycoproteins or glycolipids (175). On the other hand, fig α-galactosidase can easily hydrolyze the α-galactosyl linkages present in ceramide trihexoside isolated from various tissues (109) and a glycopeptide isolated from earthworm cuticle collagen (127).

In Cucurbita, the raffinose family of oligosaccharides are translocated into the immature leaves, and other developing areas, where they are metabolized (182,185, 195). The input of the sugars cease when the leaves mature. The mature leaves then synthesize the sugars by photosynthesis and translocate them through the vascular system. Although, the enzyme is present in mature leaves, the apparent lack in vivo activity was surprising. The enzyme may be separated from the site of sugar synthesis and from the transport route. Thus, α-galactosidase may be present as a compartmentalized enzyme.

Another possible role for the enzyme is protecting plants from α-galactosidic phototoxic substances (175) produced by invading microorganisms. Binding of the toxin is reduced in the presence of α-galactosides when attached to the membrane-bound protein ; the toxin may cause a conformational change which ultimately affects membrane permeability (172,174). The enzyme may also take part in hydrolyzing α-galactosyl containing substrates that leak through plasmalemma, enabling the free sugars to enter the cell. Perhaps it aids in degrading galactolipids and disintegrating membranes during cellwall lysis, which may occur following injury, or senescence. The role of α-galactosidase in fruits is not clear; it is probably involved in the degradation of oligosaccharides. This enzyme participates in the control of internal osmotic pressure in the unicellular alga (58).

## 2.8 REGULATION

In fenugreek and carob (142,159) α-galactosidase was synthesized by the cells of the aleurone layer and its synthesis was inhibited by cycloheximide and abscisic acid. However, in the presence of 5-fluorouracil or actinomycin-D, the enzyme level attained a point as high as that in the control. This might indicate that m-RNA species are moderately stable. The synthesis of the enzyme in carob was demonstrated by the incorporation of [U-$^{14}$C] serine into the protein (159).

Sentandreu, et al.(160) studied the effect of tunicamycin on the synthesis and secretion of two external yeast enzymes; α-galactosidase and β-gluconase. The synthesis of total active α-galac-

tosidase (internal plus external by yeast protoplasts was immediately stopped after the addition of tunicamycin, although the preexisting internal enzyme which contains reduced amount of carbohydrate was secreted. Interestingly enough, increasing concentrations of tunicamycin (10 to 50 μg/ml), which did not affect protein synthesis, did speed up the rate of secretion. In Aspergillus niger presence of tunicamycin (40 μg/ml) decreased the activities of α-galactosidase by 70% and the secreted enzymes were devoid of carbohydrate. Similarly, intracellular activities were inhibited by 50% (1,170). These results suggest that α-galactosidase is a glycoprotein enzyme and that carbohydrate moiety was essential for the enzyme activity but not for secretion.

## 2.9 PURIFICATION

The separation of the multiple forms of plant α-galactosidases has been achieved by applying methods that resolve macromolecules according to their molecular weight (gel filtration), and ionic characteristics (ion exchange chromatography, electrophoresis and isoelectric focusing; 195). The procedure for extracting the enzyme from tissues determines the level of the total enzyme as well as the multiple-form composition. For example, purification of cell-wall associated α-galactosidase from Vigna (76), in the absence of D-galactose and 2-mercaptoethanol in the buffer medium, yielded only one enzyme peak (molecular weight 160,000), which also displayed lectin activity, on gel filtration. But removal of the two additives from the buffer yielded additional peaks of lower

molecular weight proteins, which exhibited either enzymic or lectin activity. In a few cases α-galactosidases have been obtained in highly purified and homogeneous form. For instance, Suzuki *et al.*(175) have obtained the enzyme in crystalline form from the fungus *Mortierella vinacea* ; Petek *et al.*(135), isolated the enzyme in homogeneous form from *Vicia sativa* by using ammonium sulphate fractionation, selective pH precipitation, organic solvent fractionation, Sephadex gel filtration, DEAE-Sephadex and ion exchange chromatography. In the case of *V.faba* (59), use of different salt concentrations, or dissimilar buffer of varying molarities, as extracting media produced varying patterns of the multiple forms of α-galactosidase. Complications in interpreting the results arose because certain buffer ions and salts caused time dependent interconversion of the enzyme forms (59). In addition, work with *V.faba* has revealed that the physiological state of the seed may play an important role in determining the number and level of the enzyme forms (189).

The conventional methods used for purifying the enzyme are described by Dey and Pridham in 1972 (51). Affinity chromatography has proved successful for purifying several α-galactosidases (41, 178). Crude preparation of amylase and glycosidases from *Achatina fulica* hepatopancreas were purified by affinity chromatography, gel filtration and ion exchange chromatography (197). Williams Janice *et al.*(1978) have purified α-galactosidases II, III and IV from seeds of *Trifolium repens* using $(NH_4)_2SO_4$ precipitation, hydroxylapatite, Sephadex gel filtration and DEAE cellulose column

chromatography (195). Using ammonium sulphate fractionation, heat treatment and selective adsorption of α-galactosidases on red blood cells followed by Sephadex gel filtration, Li, et al.(107) achieved 100-folds purification of α-galactosidase from Pneugoccal. N-G-Aminocaproyl α-D-galactosylamine was coupled to Sepharose and used as an effective affinity column (82). In several instances, Con A-Sepharose was employed as an affinity adsorbent (46,59,61), which retarded those glycoprotein α-galactosidases having glucose/mannose termini on the protein. Other glycoproteins with similar carbohydrate moieties also bind to such affinity columns. The eluting medium in this case was methyl-α-D mannoside or glucoside. Schram and Andrew et al.used immobilized antibodies for the isolation of the lysosomal acid α-glucosidase from normal human liver. Antibodies raised against the purified enzyme were immobilized by covalent coupling to Sepharose 4B (154, 158).

Multiple molecular forms of α-galactosidase from normal and fabry leukocytes were separated using preparative electrofocusing (149,150,151). Dean, Kenneth and James purified α-galactosidases A and B from human liver, by a procedure that included hydroxylapatite and ampholyte displacement chromatography (37,38,39). Human plasma α-galactosidase A was purified 7000-fold ever plasma levels from cohn fraction IV-1 by Bishop, David et al.(18). Husted developed a new method of extraction in aqueous two phase systems for the large scale purification of enzymes (87). α-Galactosidases from Aspergillus niger have also been purified using hydroxylapatite column chromatography (9). Miura, et al.(126) used high performance liquid chromatography for simultaneous determination of multiple enzyme activities from several glycosidases.

## 2.10 GENERAL PROPERTIES OF α-GALACTOSIDASES

### 2.10.1 OPTIMUM pH AND OPTIMUM TEMPERATURE

Most plant α-galactosidases are fairly stable to pH variation (51); thus, lowering the pH of the crude extract to 3–4 is often used to precipitate storage proteins in initial steps of purification (51). The enzyme is generally stable at 4°C, but sometimes loses activity on freezing and thawing (51). Each of the multiple form of the enzyme in Cucurbita leaves displayed a distinct pH profile. Enzyme I and II were active in a narrow pH range, whereas III had a wider range. Hence, the local pH in the tissue and the relative activity of the appropriate enzyme form probably determine the rate of breakdown of the natural substrates. Enzyme III operates in a region where pH is poorly controlled. A fourth form of α-galactosidase with alkaline pH optimum was shown in young leaves and was more specific for the hydrolysis of stachyose (183). Thus it shows that α-galactosidase had a broad active range from pH 3.0 to pH 6.5 (65).

### 2.10.2. MOLECULAR WEIGHTS OF α-GALACTOSIDASES

The molecular weights of α-galactosidases vary from source to source. Among the lowest molecular weight of α-galactosidases that have been reported so far are from Helianthus annus, Ceratonia siliqua, Medicago sativa , Mr = 25,000 (11, 119). On the other hand the highest molecular weights of α-galactosidases, Mr=2,09,000 have been reported from Vicia faba seeds (49,50).With the exception of a few plant species

all contain multimolecular forms of α-galactosidases. It is not known if the enzyme of lower molecular weight(s) from an organism are precursors for those of higher molecular weights. It has now been established that among the molecular forms differentiated by their molecular weights, a monomeric/tetrameric relationship generally exists (Table II), except in cases where larger aggregates may be formed ( 139). The tetrameric enzyme from soybean was converted predominantly into the monomeric form on storage in pH 7.0 buffer at - 10°C for 10 h. However, on dialysis of the stored enzyme at pH 4.0, reconversion to the tetramer occurred (82). The tetrameric soybean enzyme (Mr≈160,000) gave two closely spaced protein bands (Mr≈40,000 and 38,000) on SDS-gel electrophoresis. It is not clear whether this observation was due to microheterogeneity of the constituent monomers or to two totally dissimilar subunits. It was, however, demonstrated for some α-galactosidases that a single subunit constituted the tetrameric glycoprotein enzyme ( 60 , 61), and that only L-alanine was present as the N-terminal amino acid (82). The microheterogeneity of the enzyme might arise from varying degrees of glycosylation of the monomeric constituent protein.

The reasons for the multiplicity of enzymes are varied. This phenomenon may be due to genetically independent proteins, genetic variants (allelic), or heteropolymers of polypeptide chains that are bound noncovalently. Conjugation of other molecules with proteins or multimers of a single subunit may also be one of the causes. Other factors, such as conformational differences between the molecules of a native protein, or covalent alteration of a native protein, also cause multiplicity of enzymes.

Aggregation of active monomeric enzymes yielding multimeric forms can be exemplified by some legume α-galactosidases. This occurred both *in vivo* and *in vitro*. Extracts of green and immature *Vicia faba* seeds showed only a low level of the monomeric form of α-galactosidases. The level of activity increased during early seed maturation, accompanied by a marked gain in specific activity (55,139). The latter increase was related to the appearance of the tetrameric form of the enzyme, which normally displays a several fold higher specific activity. Analysis of enzyme extracts of seeds in the course of maturation showed a gradual increase of the tetrameric enzyme, with a concomitant fall of the monomeric form (139). Initial germination of mature seeds showed a reversal of the process (139).

An *in vitro* conversion of the monomeric to the tetrameric enzyme of *V.faba* was also demonstrated. Extracts of seeds having only the monomeric form, on storage at 4°C for 70h in a buffer of pH 5.5, showed a 4.4-fold increase in specific activity (208,600). The stored enzyme had predominantly the tetrameric form. The process of conversion of the monomer to the tetramer could be accelerated by raising the pH of the extract to 7.0 and the temperature to 25°C. There was also evidence that the conversion of the monomer to the tetramer proceeded via an intermediate form (59). Similar *in vitro* aggregation was demonstrated in Lentil (61) in this only concentrating the enzyme solution caused aggregation.

The tetrameric lentil enzyme (61) consisted of identical subunits, however, the *V.faba* enzyme showed some microheterogeneity of the main protein band, which was obtained after SDS gel electrophoresis (60). The *V.faba* α-galactosidases are glucose-mannose containing proteins and immunologically cross-react with each other (60). The monomeric form was thought to be a microheterogenous mixture of glycoproteins existing at varying stages of glycosylation. As the enzymes were also glucose-mannose specific lectins, the aggregation was possibly aided by lectin interactions. This process perhaps preferentially selected monomers with higher carbohydrate contents, although those with lower sugar content were also able to aggregate. Thus, on analysis, the tetrameric form showed a

relatively higher carbohydrate content on a molar basis as compared to the monomeric form (60). A third form of the enzyme, other than the tetramer and the monomer, with a molecular weight slightly higher than the latter, was also detected in V.faba. This form was separated by CM-cellulose chromatography. Although the enzyme is immunologically related to the other two forms (60), its genetic origin is not known. Since some of the legume α-galactosidases possess lectin activity (60,61), it is doubtful whether the classical legume lectins are the precursors of the enzymes, or proteins that lost their enzymic activity during the course of evolution.

## 2.11 SPECIFICITY OF HYDROLYTIC ACTION

As regard hydrolytic property the enzyme can cleave α-D-galactopyranosyl residues from the non-reducing end of simple galactosides (51). Some are also able to liberate galactosyl residues from α-galactosyl containing polysaccharides (121). Most of the forms of enzyme are not absolutely specific for the glycon residue and will hydrolyse the structurally related α-D fucopyranosides and β-L-arabino pyranosides (114).

The quantitative evaluation of glycon specificity has been carried out by Dey and Pridham. These authors reported that the affinity (1/Km) of the enzymes for the substrates was dependent largely on the structural changes in the glycon moiety and follow the order α-D-galactoside > α-D-fucoside > β-L-arabinoside. From this it was concluded that one of the specific

points of attachment of the substrate to the enzyme was through the primary alcoholic group of the galactose structure (51). Among galactosides, the order of decrease in rates of enzyme hydrolysis was aryl-α-galactoside, sugars and alkyl-α-galactosides (108).

In addition, a large number of naturally occuring and synthetic glycosides are known to be hydrolysed by various α-galactosidases. Galactomannans, which normally have a basic structure consisting of a bulk bone of β-1, 4-linked D-mannosyl residues to which D-galactosyl residues are attached by α-1, 6-linkages are attacked by α-galactosidases from various sources (10).

## 2.12 GALACTOSYL TRANSFERASE ACTION

α-galactosidases also show trans-α-galactosylation activit in addition to hydrolytic activity, and the products are comple carbohydrates (51). As shown in figure 1 water can be replaced by a number of organic galactose acceptors and in this way simple galactosides and oligosaccharides have been synthesized. Melibiose could also be formed *in vitro* by almond α-galactosidase catalyzed transglycosylation to D-glucose as an acceptor (55).

The equilibrium for a α-galactosidase catalysed reaction, as with other glycosidases, normally favours hydrolysis. In the case of an enzyme isolated from *Phaseolus Vulgaris* by Tanner and Kandler (180), however, transgalactosylation from

galactinol to raffinose (with the formation of stachyose) occurs more readily than the hydrolysis of these galactosides. These workers have shown that the involvement of inositol in the formation of glycosidic linkages was an essential step for the biosynthesis of oligosaccharides in the raffinose series (106, 180). De novo synthesis of oligosaccharides is also possible when high concentrations of galactose are incubated with the enzyme (51). Li, et al.(107) showed that α-galactosyl groups from suitable oligosaccharides (melibiose and raffinose) are transferred to primary and secondary alcoholic groups. The transfer reaction brought about predominant formation of α-(1-6) linkage.

## 2.15 EFFECT OF SULPHYDRAL SPECIFIC REAGENTS

α-galactosidase from Aerobacter aerogenes can be inhibited by 'sulphydryl reagents', such as p-chloromercuribenzoate, N-ethyl maleimide, and iodoacetamide (86). Sulphydryl reagents also inhibit α-galactosidases from Diplococcus pneumoniae (107) and Bacillus stearothermophillus (132). On the other hand α-galactosidases from Calvatia cyanthiformis (108) and Mortierell vinacea (175) are not specifically inhibited by such reagents. Coffee beans α-galactosidase was inhibited strongly with p-chloromercury benzoic acid, but is not significantly affected by other metal ions, SH-reagents and EDTA (115). Pure Na-taurocholate at increasing concentration inhibited α-galactosidase 'A' with p-nitrophenyl-α-galactoside as substrate (155,157).

Triton X-100 had no activator or inhibitor effects on either enzyme activity, but almost completely solubilized these enzymes (150) obtained from human tissue (150). Thus, not all α-galactosidases require - SH groups for activity.

## 2.14 EFFECT OF HEAVY METAL IONS

Various metal ions also inhibit the enzyme (51). Among the most extensively studied heavy metal ions are : $Ag^{+}$ (72,108,115), $Cu^{2+}$ (107) and $Hg^{2+}$ (107,108,115). It is interesting to note that while a particular metal ion is highly potential inhibitor of α-galactosidase from a source, it is either ineffective or a poor inhibitor for the same enzyme from another source. For instance, α-galactosidase obtained from Spinacia olervaceus (72) was potentially inhibited by $Ag^{+}$ at $10^{-6}$M concentrations, but the one from Calvatia cyanthiformis (108) was not at all inhibited at $10^{-5}$M concentrations. Similar variation in the degree of the inhibition of α-galactosidases obtained from different sources has been reported for $Cu^{2+}$ and $Hg^{2+}$ also. It may be pointed out, however, that ineffectiveness of $Ag^{+}$ and $Cu^{2+}$ ions towards the α-galactosidase from C.cyanthiformis could be due to the presence of cyanoproteins which prevent the interaction of $Ag^{+}$ or $Cu^{2+}$ with α-galactosidase by making silver or copper cyano complexes.

In general, the inhibition of α-galactosidases by $Ag^{+}$, $Cu^{2+}$ and $Hg^{2+}$ has been reported to be of competitive nature (51). On the other hand Thorpe (185) investigated that $Zn^{2+}$ significantly stabilized human splenic α-galactosidase 'A' against thermal denaturation at pH 6.5.

## 2.15 EFFECT OF SUGARS AND SUGAR ALCOHOLS

D-galactose is a competitive inhibitor of α-galactosidase (51,70,108,175). Higher concentrations of substrate are also inhibitory to the enzyme. The competitive inhibition by galactose, is of course, a case of product inhibition. The structural analogues of D-galactose, that is, L-arabinose, D-fucose, D-arabinose, cellobiose, melibiose (132,175). 2-deoxy glucose, 2-deoxy-D.galactose, D-glucose, D-mannose, D-fructose and D-ribose do not produce inhibition of the α-galactosidase that have so far been examined (36,108,175). For attachment of sugar to the enzyme, D-galactose configuration is required and $C_1$, $C_2$, $C_4$ and $C_6$ are involved in binding. Sharma (162) has shown that a highly specific inhibition of α-galactosidase with myo-inositol was due to the similarity in the orientation of - OH groups at $C_2$ , $C_3$ and $C_4$ in myoinositol to those at $C_4$ , $C_3$ and $C_2$ or $C_1$ , $C_2$ and $C_3$ of the α-D-galactopyranosyl residue of the substrate. The -OH group of $C_4$ of D-galactose seems to uniquely satisfy both the spatial and H-bonding requirements of the activated enzyme from Aspergillus fumigatus because analog of D-galactopyranoside modified at $C_4$ was a strong inhibitor of the enzyme (166).

Among sugar alcohols arbitol was found to be an effective inhibitor of α-galactosidases obtained from chickpea seeds while even the higher concentrations of Xylitol and galactitol produced no detectable effect on it (163). In Mortierella vinacea substrate inhibition diminished gradually with increas-

ing temperature or neutral pH range while with decreasing temperature or in acidic pH range substrate inhibition was accelerated (99). Burstein and Kepes (26) have reported that α-galactosidase of E.coli $K_{12}$ loses activity if stored in Tris. It is interesting that α-galactosidases are not inhibited by D-galactono 1,4-lactone (154). In the case of other glycosidases, however, corresponding aldonolactones are strong competitive inhibitors.

α-Galactosidases from several sources display a unique activity namely, agglutination (60,75,76,78,154,158,186). In mung bean, only the tetrameric form of the enzyme displayed the hemagglutinating activity (77). It provided the appropriate number of binding sites for binding the α-galactosyl residues to t the blood cell surfaces to cause agglutination. In contrast the monomeric form has only one binding site available, which is not sufficient to cause agglutination of blood cells. Lectin/α-galactosidases with similar properties were found in seeds of four other legume species (79). These proteins appear to belong to a family of immunologically related α-galactosidases found in a large number of legumes; Only few of these plants show hemagglutinating activity (28,80). Similarily a protein which is devoid of lectin activity was isolated from stem and leaves of Dolichos biflorus was found to be immunologically cross-reactive with the seed lectin from the same plant(177). Kauss, thought that this material possibly represented a

precursor of the lectin (95). Soybean α-galactosidase converts human type 'B' erythrocytes to type 'O' (82) by cleaving the non-reducing terminal α-D-galactosyl residues of blood group B antigen. Harpaz and coworkers (82) were unable to observe hemagglutination using purified soybean α-galactosidase, probably because the enzyme activity was high enough to cause clot formation and dissolution simultaneously at the pH of the assay. These observations and inhibition studies with several saccharides indicate that the carbohydrate binding site of the lectin and α-galactosidase have a rather similar or identical carbohydrate specificity. Haass, et al. have (76) observed that α-galactosidase remains together with the lectin through all the purification steps, indicating that both activities might be present in the same molecule. However, it was also demonstrated (76) that part of the wall associated lectin/α-galactosidase can be eluted by salt solutions other than the complexing phosphate buffer. In Vicia faba the enzyme may be present in a conjugated form in combination with the classical glucose/mannose specific lectin (5) and hence display an overall lectin activity.

## 2.16 GLYCOPROTEIN NATURE

Several legume α-galactosidases have been shown to be glycoproteins with affinity for Con A (60,61,77). The glycorprotein nature may be a common feature among the seed enzymes of this family (62). The glycopeptide from the lentil

enzyme has been isolated (61) and tentatively identified as-

$$(Man)_7 - Glc\ NAc - Glc\ NAC - \begin{array}{c}|\\ Asn\\ |\end{array}$$

A similar structure was proposed for the glycopeptide from A.niger α-galactosidase (1).

From the literature survey presented here it is apparent that although a large body of data is available on α-galactosidases, there are a number of vital questions which still remain unanswered. For instance information on the physiological significance of the multimolecular forms or the structural, biochemical and genetical relationship between various forms of α-galactosidase in plants is highly inadequate.

## 3.0 EXPERIMENTAL PROCEDURES

### 3.1 MATERIAL

P-Nitrophenyl α-D-galactopyranoside, used as a substrate, was purchased from Pierce Chemical Co., Rockford, Ill, (U.S.A.). Sodium dodecyl sulphate (SDS), myoinositol, Sephadex $G_{150}$, bovine serum albumin (BSA) and CM-cellulose were purchased from Sigma Chemical Co., St.Louis, Missouri (U.S.A.) Acrylamide, N-N'-methylene-bis acrylamide, and N,N,N',N' tetramethyl ethylenediamine were obtained from Serva, Feinbiochemica, Heidelberg, (Germany). Folin and Ciocalteu's reagent (phenol reagent), enzyme grade sodium chloride and ammonium sulphate were obtained from E.Merk, India. The Protein standards for molecular weight determination were obtained from Bio-Rad (U.S.A.). Sodium alginate was purchased from Alginate Industries Limited, Girvan, Ayrshire. All other chemicals used in the present study were of analytical reagent grade from various commercial sources.

Cicer-arietinum seeds (white and black varieties) were purchased from the local seed stores. The variety $H_{208}$ and $C_{235}$ were obtained from Indian Agriculture Research Institute, New Delhi, (India).

### 3.2 METHODS

#### 3.2.1 ENZYME ASSAY

α-galactosidase activity was measured with p-nitro phenyl α-D-galactopyranoside as substrate by the same method as described by Bahl and Agarwal (8). The reaction mixture

contained the following in the final volume of 1 ml:100µmoles of substrate, 0.1-0.2 ml of enzyme solution (10-70 µg protein depending upon the form of enzyme assayed) and 50 mM sodium acetate buffer, pH 5.2. The enzyme action was started by addin the buffered substrate solution to the reaction mixture. Incub tion was carried out at 25°C for 15 min. Controls, both withou substrate or without enzyme, were incubated concurrently with the samples. The enzyme activity was terminated by adding 1.5 ml of 4% (w/v) sodium carbonate solution. The liberated p-nitrophenol, which produced yellow colour in alkaline pH, wa measured at 420 nm by Bausch and Lomb spectronic-20 spectrophotometer. The amount of p-nitrophenol released was determine by referring to a standard curve prepared concurrently in the same manner with 10-100 µmoles of p-nitrophenol.

One unit of enzyme was defined as the amount of protein in mg which liberated one µmole of p-nitrophenol per minute at 25°C. The specific activity of the enzyme was expressed as the number of units per mg of protein.

3.2.2 PROTEIN ASSAY

Protein was estimated by the method of Lowry et al.(68). A standard curve of protein was prepared by using varying amounts of bovine serum albumin (10-100 µg). The colour was developed with Folin and Ciocalteu reagent (1:2 dilution v/v) and the colour density was measured at 690 nm. The amount of protein in the enzyme sample was computed by referring to standard protein curve prepared concurrently.

### 3.2.3 GERMINATION OF SEEDS

Chickpea seeds (Cicer-arietinum) were germinated in dark at 25°C under asceptic conditions. Surface sterlization of healthy seeds was done by treatment with 0.1% sodium hypochlorite solution for 5 minutes, followed by a thorough washing with water to remove the hypochlorite completely. Seeds were then spread on 4 layers of cheesecloth in a tray and soaked with water and allowed to germinate. Throughout the germination period the seeds were washed twice a day with water to prevent any fungal infection. After germinating for specified period of time cotyledons were separated from the seedlings and used for enzyme extraction.

### 3.2.4 PREPARATION OF ENZYME EXTRACT FOR ROUTINE ANALYSIS

All the operations were carried out at 0-4°C unless otherwise stated. Germinating cotyledons (200g) were homogenized in prechilled 2 lit of 50 mM acetate buffer, pH 5.2, using a Waring blender for about 1 min. The homogenate was centrifuged at 12,100 X g for 30 min. The clear homogenate was subjected to pH precipitation. At pH 3.0 a significant amount of protein precipitated. The precipitate was separated from the supernatant by centrifugation and was discarded since it contained no α-galactosidase activity. The clear supernatant was then brought to pH 5.2 by the slow addition of 1 M sodium acetate solution. To the supernatant $(NH_4)_2SO_4$ was added in order to achieve 30% saturation. The protein fraction

precipitated was removed by centrifuging at 50,000 X g for 60 min. Since this fraction was devoid of the α-galactosidase activity, it was discarded. To the supernatant sufficient amount of $(NH_4)_2SO_4$ was added in order to achieve 70% saturation and the protein precipitate was separated by centrifugation. It was dissolved in minimal volume (2-5 ml) of 50 mM sodium acetate buffer, pH 5.2, dialyzed overnight against 3 x 200 ml of the same buffer. Almost total activity of α-galactosidase was found to be present in dialyzed fraction. It was used in prepurification and developmental studies of the total α-galactosidase activity.

3.2.5 PREPARATION OF ACETONE POWDER

Chickpea cotyledons (200g) were homogenized in Waring blender with 1 litre chilled acetone (-10°C) for 2-5 min. The homogenate was filtered through Buchner funnel and washed several times with chilled acetone. The residue was spread on a thick sheet of Whatman filter paper and allowed to dry in open air at room temperature (20°C). In order to avoid variations in samples, sufficient quantity of the acetone powder was prepared from a batch of seeds to be used as starting material for the enzyme isolation, purification and characterization work. The dry acetone powder could be stored at 4°C in an air tight desiccator for several months without any loss of α-galactosidase activity. The acetone treatment was found useful in the removal of fats, pigments, gums and some resin like material which would otherwise create difficulties in the subsequent purification steps.

### 3.2.6 pH-PRECIPITATION

Acetone powder ( 15 g) was extracted with 150 ml of 50 mM sodium acetate buffer, pH 5.2 for 12 hour. The homogenate was filtered through four layers of cheesecloth and centrifuged for 30 min at 12,000 X g. The clear cell free supernatant was collected and subjected to pH precipitation. To the enzyme extract prechilled 1 M acetic acid was added to bring the pH of the crude extract to 3.0, and the solution was allowed to stand for 3 h in ice bath for complete precipitation. The precipitate was then separated from the supernatant by centrifuging it for 30 min at 50000 X g. The pellet was discarded, since it contained no α-galactosidase activity. The clear supernatant was then brought to pH 5.2 by the slow addition of chilled 1 M sodium acetate solution. At this stage no precipitate was observed and the enzyme remained in the solution.

### 3.2.7 AMMONIUM SULPHATE FRACTIONATION

For further purification the clear supernatant obtained after pH precipitation was subjected to ammonium sulphate fractionation. To the enzyme extract sufficient amount of ammonium sulphate was added gradually with constant stirring to obtain 80% saturation. It was kept overnight at 4°C and then centrifuged at 50000 X g for 60 min to remove the protein precipitate. The protein fraction was found to have no α-galactosidase activity. Further addition of ammonium sulphate

to the supernatant was carried out, so as to bring 70% saturation. The precipitate formed was allowed to settle down for at least 6-7 h at 4°C and then centrifuged as before. The protein residue so obtained was quite rich in α-galactosidase activity, the supernatant did not show any α-galactosidase activity and was therefore, discarded. The protein fraction was dissolved in minimal volume (5-10 ml) of 50 mM acetate buffer, pH 5.2, dialyzed for 24 h against same buffer and tested for α-galactosidase activity. The dialyzed fraction was found to be highly rich in α-galactosidase activity.

3.2.8 GEL FILTRATION ON SEPHADEX G-150

Sephadex G-150 (particle size 10-40 μ, water regain 25 ± 1.5, bed volume 20 ml/g) was suspended in 50 mM sodium acetate buffer, pH 5.2, and allowed to swell for 4-5 days at room temperature (25°C). The floating fine particles were removed by repeated decantation. The gel was diluted with the same buffer and deaerated under reduced pressure with the help of a water suction pump and packed under gravity in a double walled glass jacketed column (2 X 100 cm) fitted with glass wool at bottom to a column height of 80 cm. The column was fully equilibrated with the elution buffer, 50 mM sodium acetate buffer, pH 5.2 for about 24 h at a flow rate of 6 ml/h or until the pH of the buffer at the entry and the buffer discharged from the column, was same. A filter paper disc of Whatman No.1 was placed on the top of the column in order to prevent any disturbance which could arise

during the application of sample. The temperature of the column was maintained at $4^{o} \pm 2^{o}C$ by circulating ice cold water in outer Jacket of column.

A 3 ml sample (125-175 mg enzyme protein) obtained after dialysis of 30 - 70 per cent ammonium sulphate fraction was applied to the previously cooled and equilibrated column. When the enzyme sample got absorbed into the column, the residual enzyme material was washed down into the gel body with 1-2 ml of additional elution buffer. The enzyme protein was then eluted with the same buffer. Two ml fractions were collected at a flow rate of 6 ml/h. Aliquots (0.1 ml) from every other fractions were analysed for protein contents and α-galactosidase activity. Two peaks of enzyme activity were obtained. These were referred to as α-galactosidase I (high molecular weight) and α-galactosidase II (low molecular weight).

The fractions with α-galactosidase activity were pooled separately, brought to 100 per cent saturation with $(NH_4)_2SO_4$ and centrifuged for 60 min at 50000 X g. The precipitate so obtained was dissolved in 2 ml of 50 mM acetate buffer, pH 5.2, and dialysed against the same buffer for 12 h.

3.2.9 DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

The DEAE-cellulose (10 g ) ion exchange resin was washed well with water, charged with 0.5 N NaOH and 0.5 N HCl successively, and finally washed with deionized water until

the effluent was neutral. The charged DEAE cellulose was suspended in 500 ml of 0.01 M phosphate buffer, pH 7.2 and packed in a glass column (2 X 50 cm) to a final height of 25 cm. The column was fully equilibrated by washing it overnight with the same buffer at a flow rate of 0.2 ml/min.

A 5 ml aliquot(15-20 mg protein) of the dialysed fraction from Sephadex G-150 was applied to the column. The unabsorbed proteins were removed by washing the column with 15 ml of 0.01 mM phosphate buffer, pH 7. The absorbed protein were eluted by passing successively 25 mM, 50 mM, 100 mM, 200 mM, 300 mM, 400 mM and 500 mM sodium chloride (20 ml each in 0.01 M phosphate buffer, pH 7.2. The flow rate was 12 ml/h and 1 ml fractions were collected. Aliquots (0.2 ml) from every other fractions were analysed for protein and the enzyme activity.

3.2.10 CM-CELLULOSE COLUMN CHROMATOGRAPHY

For further purification the α-galactosidase fractions obtained from Sephadex gel filtration were subjected to ion exchange chromatography on CM-cellulose column (1 X 20 cm). The CM-cellulose was charged by the following procedure before filling in the column. Fifteeng of material was suspended in water and fine particles were removed by repeated decantations. The resin was filtered on a Buchner funnel and allowed to swell with occasional slow stirring in 500 ml of 1N HCl. After 30 min, it was filtered again on a Buchner

funnel, rinsed with deionized water and resuspended in 500 ml solution of 1N NaCl and 1N NaOH (1 : 1 v/v). After 10 min it was again filtered and thoroughly rinsed with glass distilled water. The material was equilibrated in starting buffer (50 mM acetate buffer, pH 5.2) and packed in Jacketed column (1 X 50 cm) upto the height of 20 cm. The column was washed overnight with the same buffer. The fraction (17 - 25 mg) from Sephadex column was applied to this column and the unabsorbed protein was first eluted with 40 ml 50 mM acetate buffer at a flow rate of 12 ml/h. The absorbed protein was eluted by the linear gradient of NaCl from 0-700 mM using 100 ml of 50 mM acetate buffer, pH 5.2, in the mixing chamber and 100 ml of 700 mM NaCl solution in 50 mM acetate buffer, pH 5.2, in the reservoir. Fractions (2 ml) were collected at a flow rate of 12 ml/h. Each fraction was analysed for protein and enzymatic activity. Tubes containing enzyme activity under individual peaks were pooled.

### 3.2.11 POLYACRYLAMIDE GEL ELECTROPHORESIS

#### 3.2.11.1 POLYACRYLAMIDE GEL ELECTROPHORESIS WITHOUT SDS

The homogeneity of the purified enzyme fractions was tested by polyacrylamide gel electrophoresis (PAGE). The procedure of Gabriel (71) was followed for discontinuous polyacrylamide gel electrophoresis using gel rods (9 cm length X 0.5 cm diameter). The electrophoretic mobility with respect

to bromophenol blue was measured by the following equation:

$$R_f = \frac{A \times l}{B \times l'},$$

where,

$R_f$ = Electrophoretic mobility ratio.

A = Distance of protein migration from origin.

B = Distance of bromphenol migration from origin.

l = Gel length before staining.

l' = Gel length after staining.

Various solutions used were as following:

Stock Solutions

Solution-A

| | |
|---|---|
| 1M Phosphate buffer | 10 ml. |
| TEMED | 0.23 ml. |

Total volume was made to 100 ml with distilled water.

Solution-B

| | |
|---|---|
| Acrylamide | 60.0 g |
| N,N'-methylene-bis-acrylamide | 0.6 g |

Total volume was made to 100 ml with distilled water.

Solution-C

| | |
|---|---|
| Ammonium persulfate | 140 mg |
| Distilled water | 100 ml |

Working solution for preparation of gel

| Solutions | 7.5% gel (separating gel) |
|---|---|
| Solution - A | 1.0 Volume |
| Solution - B | 1.0 Volume |
| Solution - C | 4.0 Volume |
| Distilled water | 2.0 Volumes |
| | 4% gel (stacking gel) |
| Solution - A | 2.0 Volumes |
| Solution - B | 1.0 Volume |
| Solution - C | 8.0 Volumes |
| Water | 4.0 Volumes |

PREPARATION OF GEL COLUMNS

Before adding the solution 'A' separating gel solution and stacking gel solution were deareated to remove the dissolved air. To the deareated separating gel solution required volume of solution 'A' was added, mixed well and immediately pipetted upto a fixed height (7-8 cm) into the gel glass tubes, placed in a vertical position with lower end closed. A drop of buffer was also added on the top of the gelling solution to avoid formation of concave meniscus. Gels were allowed to polymerize for 2 h . After complete gel formation the buffer from the top of the gel was removed and the deareated stacking gel solution was laid over the gel column to a height of 0.5 cm.

RUNNING BUFFER

In the case of experiments concerning electrophoretic mobility of enzyme, 0.05 M sodium phosphate buffer, pH 7.5, was used. To test the homogeneity of the purified enzymes electrophoresis was run at three different pH i.e. pH 6, pH 7.5 and pH 8.

PREPARATION OF SAMPLES

Samples of enzymes (100 µg protein) from CM-cellulose column were dialyzed against distilled water and dried. Residual proteins were dissolved in 0.01 M phosphate buffer, pH 7.0, containing 0.001% bromophenol blue and 40% sucrose solution.

ELECTROPHORESIS

The tubes with gel columns were fixed in the electrophoretic apparatus and the running buffer was filled in the anode and cathod chambers of the apparatus. Individual protein samples (40-100 µg) were layered on the top of the separate gel columns. The electrophoresis was carried out at a constant current of 6 mA per tube until the tracker dye, bromophenol blue, migrated to the lower end of the gel columns. The operation required about 2.5 h for completion. The gels were taken out from glass columns and stained by immersing in 0.1% Coomassie Blue R-250 in 10% acetic acid, 25% methanol and water (v/v/v) for 6-8 h at room temperature. The gels were destained by using a solution of 25% methanol, 10% acetic acid and distilled water.

3.2.11.2 SODIUM-DODECYL-SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed in the presence of 0.1% SDS in 0.1 M phosphate buffer, pH 7.0 as described by Laemmli (101). The gels were prepared using the PAGE stock solutions in the following manner :

| Stock Solutions | 10% gel (separating gel) |
|---|---|
| Solution A with 0.2% SDS | 4 Volumes |
| Solution B | 5 Volumes |
| Solution C | 16 Volumes |
| Water | 7 Volumes |

RUNNING BUFFER

In case of experiments concerning molecular weight determination or subunit structures 0.05 M phosphate buffer, pH 7.5, with 0.1% SDS was used as electrophoresis buffer.

PREPARATION OF SAMPLES

The samples were prepared by the method given in Bio-Rad laboratories, Technical bulletin (17). The enzyme from CM-cellulose column was dialyzed against distilled water and dried. The protein thus obtained was dissolved in the sample buffer of the following composition:

0.01 M sodium phosphate buffer (pH7); 1% sodium dodecyl sulphate (SDS); 0.1 M dithiothreitol or β-mercaptoethanol; 40% sucrose ; and 0.001% bromophenol blue.

All the protein samples were then denatured by incubation at 100°C for 5 min.

ELECTROPHORESIS

Protein samples and standard protein mixture (40-100 µg protein) were layered on gels through the electrophoresis buffer. The electrophoresis was carried out at a constant current of 6 mA per tube for 2.5 h at room temperature. The protein bands were located by staining the gels with 0.1% Coomassive Brilliant blue R-250 in acetic acid/methanol/water (10:25:65 v/v/v) for 6-8 h and were destained with 10% acetic acid. Mobilities were determined relative to bromophenol dye.

3.2.12 DETERMINATION OF MOLECULAR WEIGHT BY SEPHADEX GEL FILTRATION

In order to determine the molecular weight of α-galactosidase isoenzymes, a column (1 X 80 cm) was packed with Sephadex G-150 according to the procedure outlined earlier. It was equilibrated with 0.05 M sodium acetate buffer, pH 5.2. Suitable samples of bovine serum albumin (Mr=66,500), cytochrome C (Mr=12,400) and alkaline phosphatase (from calf intestine, Mr=100,000) (5 mg each) were dissolved in the same buffer and applied to the column. Proteins were eluted with the same buffer at a flow rate of 6 ml/h . Two ml fractions were collected. Each fraction was analysed for protein contents by Lowry method (68). The elution volumes/molecular weights for each of the proteins used were determined. A standard curve was prepared by plotting the elution volume versus the $\log_{10}$Mr of the standard proteins. A linear relationship was obtained. Elution experiments with α-galactosidase

isoenzymes were also performed using the same column under identical experimental conditions. The elution volumes for both enzyme fractions were obtained and their molecular weights calculated by referring to standard curve.

3.2.13 DETERMINATION OF MOLECULAR WEIGHT BY SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

In order to determine the molecular weights of α-galactosidase isoenzymes, gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the procedure described by Laemmli (101). The standard proteins mixture for electrophoresis supplied by Bio-Rad Labs., Richmond, California, was also dissolved in the sample buffer having 1% SDS, 0.1 M β-mercaptoethanol, 40% sucrose, and 0.001% bromophenol blue in 0.01 M sodium phosphate buffer pH 7. The reference proteins were also denatured by incubation at 100°C for 5 minutes. The electrophoresis of reference proteins was also carried out along with the purified α-galactosidase isoenzymes samples.

A curve between the molecular weights and the electrophoretic mobilities of standard proteins was plotted. This curve was used as a standard calibration curve for determining the values of molecular weights corresponding to the measured electrophoretic mobilities of different isoenzymes (192).

The molecular weights of different standard proteins used were as follows:

| Proteins | Molecular weight (Daltons) |
|---|---|
| Lysozyme | 14,400 |
| Soyabean trypsin inhibitor | 21,500 |
| Carbonic anhydrase | 31,000 |
| Ovalbumin | 45,000 |
| BSA | 66,200 |
| Phosphorylase B | 92,500 |

3.2.14 DETERMINATION OF APPARENT INHIBITION CONSTANT ($K_i$)

Values of apparent inhibition constant ($K_i$) were obtained from Dixon plots of reciprocal velocity against variable inhibitor concentration. In this method rates of the enzyme catalyzed reactions were separately measured at two fixed levels of substrate concentration (0.1 mM and 0.2 mM p-nitrophenyl-α-D-galactopyranoside) in presence of variable inhibitor concentrations. In both sets of experiment levels of variable inhibitor concentrations (I), were kept constant. The results of the two sets of reciprocal velocities so obtained, were plotted against the inhibitor concentrations, resulting in two straight lines which intersected at point above the ordinate. From the point of intersection a perpendicular was drawn on the abscissa and the point of intersection gave the value of $K_i$ .

### 3.2.15 DETERMINATION OF THERMAL STABILITY

The thermal stability of the isoenzymes was determined by preincubating the enzyme solution without substrate in 0.05M sodium acetate buffer of optimum pH at indicated temperatures varying from 15° to 60°C for 30 min. After incubation, enzymes were quickly cooled in ice bath to arrest the thermal denaturation state of the enzyme and then assayed in a standard way at 25°C as described before. The activity in the control (without preincubation) was used as 100 per cent and the results are expressed as the per cent residual activity as a function of temperature.

The thermal stability of the α-galactosidase isoenzymes was also determined by preincubating the enzyme solution without substrate in 0.05M sodium acetate buffer of optimum pH at 50°C for different periods ranging from 2.5 min to 30 min in the same way described above. The results are expressed as per cent of maximum activity versus time of heat treatment at 50°C.

### 3.2.16 DETERMINATION OF APPARENT MICHAELIS MENTEN CONSTANT ($K_m$) AND MAXIMUM VELOCITY ($V_{max}$)

The values of $K_m$ and $V_{max}$ for each of the α-galactosidase isoenzymes were evaluated from Lineweaver Burk plots (112). Measurement of enzyme activity for these

plots was made at optimum pH and temperature, with different substrate concentrations varying from 100 µmol to 700µmol for each plot. These levels of substrate concentrations give from partially to fully saturated enzyme system. Reaction velocity at different substrate concentrations was determined by measuring the amount of p-nitrophenol liberated per unit time. Linear plots of reciprocals of velocity (1/V) versus reciprocals of substrate concentration (1/S) were obtained. The intercepts on 1/S axis and 1/V axis were equal to $-1/K_m$ and $1/V_{max}$, respectively.

3.2.17 DETERMINATION OF HILL'S COEFFICIENT (n)

Interaction coefficient or Hill's coefficient(n) (176) for the interaction between p-nitrophenyl α-D-galactopyranoside and the enzyme was determined by Hill's equation. The logarithmic form of the equation is given below :

$$\log\left(\frac{V}{V_{max}-V}\right) = n \log [S] - \log K_s$$

where V is the rate of reaction at a substrate concentration [S]; $V_{max}$ is the reaction rate when the enzyme is fully saturated with substrate; 'n' is interaction coefficient(number of substrate molecules bound to enzyme molecules) and $K_s$ is the binding constant.

Plots of log[S] versus log $(\frac{V}{V_{max}-V})$ showed a linear relationship. The slope of the curve equals to n.

3.2.18 DETERMINATION OF THERMODYNAMIC PARAMETERS

Thermodynamic parameters, energy of activation ($E_a$), enthalpy change ($\Delta H$), free energy change ($\Delta G$) and entropy change ($\Delta S$) were determined as described by Irshad and Sharma (88), using $K_m$ and $V_{max}$ values obtained from the Lineweaver Burk plots at different temperatures. The $K_m$ was assumed to represent the association constant for the formation of active enzyme-substrate complex.

3.2.18.1 ENERGY OF ACTIVATION ($E_a$)

The energy of activation for the α-galactosidase catalyzed reactions was determined from Arrhenius plots according to the following equation :

$$\log_{10} V_{max} = \log_{10} A - \frac{E_a}{2.303\ RT}$$

where A being the Arrhenius constant; T, the temperature in degree absolute, R, the gas constant (R=1.987 cal. $mole^{-1}$). $V_{max}$, the maximum velocity and $E_a$ the activation energy. Arrhenius plots were obtained by plotting log $V_{max}$ against 1/T. From the slope of the curve, the value of energy of activation was calculated from the following equation :

$$E_a = -2.303\ R\ X\ slope$$

### 3.2.18.2 ENTHALPY CHANGE ($\Delta H$)

The value of $\Delta H$ for the enzyme catalyzed reaction was calculated according to Van't Hoff equation:

$$\log_{10} K = A - \frac{\Delta H}{2.303\ RT}$$

where, A is a constant factor related to molecular collision and probability of reaction; R, gas constant; T, the absolute temperature and K, the dissociation constant of the enzyme substrate complex. In this equation Michaelis Menten constant, $K_m$, was assumed to be equal to K. The plots of $\log_{10} K$ versus $\frac{1}{T}$ will be linear with a slope of $-\Delta H/2.303\ R$ from which the value of $\Delta H$ was calculated.

### 3.2.18.3 FREE ENERGY CHANGE ($\Delta G$) AND ENTROPY CHANGE ($\Delta S$)

$\Delta G$ for the enzyme catalyzed hydrolysis of p-nitrophenyl α-D-galactopyranoside was estimated according to the following equation:

$$\Delta G = -2.303\ RT \log_{10} K_m$$

The terms in this equation have their usual meanings. $K_m$ is Michaelis-Menten constant assuming that it represents association constant for the formation of enzyme-substrate complex. Finally entropy change ($\Delta S$) for the formation of above complex was calculated according the following equation:

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

### 3.2.19 ANALYSIS OF GLYCOPROTEIN SUGARS

#### 3.2.19.1 REMOVAL OF N-GLYCOSIDICALLY LINKED OLIGOSACCHARIDE FROM GLYCOPROTEIN ENZYME

The glycoprotein sample (200-500 µg) was treated with 0.5-5 units N-acetyl-β-D glucosaminidase (sigma chemicals Co.) in 0.1M sodium citrate buffer, pH 7.4. Incubation was carried out at 37°C for 20 h. The reaction was stopped by placing the reaction vessel in boiling water bath for 5-10 min followed by the addition of 10% trichloroacetic acid. After cooling the denatured protein precipitate was separated from the soluble part by centrifugation at 50,000 Xg for 60 min. The precipitate was washed two times with 5% trichloroacetic acid and the supernatants from various runs were pooled. The carbohydrate content in both precipitate and the supernatant were determined. The decrease in the carbohydrate content of the acid precipitated protein and the corresponding increase in the carbohydrate content of the supernatant indicated the removal of carbohydrate by endo-N-acetyl-β-D-glucosaminidase.

#### 3.2.19.2 NEUTRAL SUGARS

Oligosaccharide, released by endo-N-acetyl-β-D-glucosaminidase treatment, was hydrolyzed as described by Tashiro and Trevithick (187) with slight modification. Oligosaccharide sample (200-500 µg) was hydrolyzed with 0.5 ml of 4N HCl for 6h in a sealed tube at 100°C. The

hydrolysate was evaporated to dryness under the stream of nitrogen many times to remove remaining HCl. The resultant residue was dissolved in 10 ml water and treated with mixed bed resin, Dowex 50X8 ($H^+$) and Dowex-1 ($Cl^-$) or Bio Rad ion-retardation resin. The resin was removed by filtration through $G_4$ glass filter and washed two times with 10 ml water. The combined filtrate was evaporated to dryness in vacuo. This fraction represents the neutral carbohydrate. The resin after removal of neutral carbohydrates was packed in a column and eluted with 50 ml of 2N HCl. The eluted solution contained amino acids and hexosamine. Both carbohydrate and hexosamine fractions were analysed qualitatively by chromatography on Whatman No.I paper with ethylacetate pyridine-water (10:4:3,v/v) and was also analysed by HPLC. To determine the amount of degradation of neutral sugars, during the course of hydrolysis, 0.5 mg of enzyme plus 50,000 cpm of [$^{14}C$]-mannose was hydrolysed and tested as described above. After paper chromatography the mannose was eluted with water directly into the counting vial, dried and counted in 5 ml dioxane cocktail fluid with Beckman LS-1801 liquid scintillation counter. The radioactivity thus eluted was compared to that of [$^{14}C$] mannose subjected to all but acid hydrolyzing conditions. The ratio of acid

hydrolyzed [$^{14}$C]-mannose to unhydrolyzed [$^{14}$C]-mannose yielded an estimate of per cent degradation of mannose (147). The correction factor was applied to both glucose and mannose. For hexosamines, 0.5 mg glycoprotein sample plus 50,000 cpm of [$^{14}$C]-N-acetyl glucosamine was hydrolyzed with 4N HCl at 100°C as before. The hydrolysate was dried under vacuum many times to remove the remaining HCl. The dried sample was redissolved in 1 ml of water and the drying step was repeated three times to ensure complete removal of HCl. The amino sugar was absorbed on Dowex 50 X 8 ($H^+$) column (1 X 10 cm) which had been previously washed with HCl, followed by deionised water. The column was washed with 25 ml of water to remove neutral carbohydrates. Then the column was washed with 50 ml 2N HCl to obtain amino sugar. The amino sugar was qualitatively analysed by paper chromatography in n-butanol-pyridine-water (6 : 4 : 3, v/v) (147). The sugar spots were located both by staining with silver and by radioactivity on the chromatogram. The comparision of radioactivity of the hydrolyzed [$^{14}$C]-N-acetylglucosamine with unhydrolyzed, yielded the extent of degradation of hexosamine during the hydrolysis. The correction was applied.

3.2.19.3 ANALYSIS OF SUGARS BY HPLC

The high performance liquid chromatography was carried out with Shimadzu LC-4A HPLC equipped with a uv spectrophotomatric detector SPD-2AS and a chromatopac

C-R2AX data processing system. The sugar samples were prepared as described by Jentoft (91). The hexosamines were reacetylated by dissolving the sample in 0.1 ml dry methanol followed by addition of 40 µl pyridine and 40 µl of acetic anhydride and incubating the sample for 1h at room temperature. The samples were dried under the stream of nitrogen. 40 µl of toluene was added and samples were redried and dissolved in water or $CH_3CN$:$H_2O$ (90:10), v/v. Sample (10 µl) was injected to a 25 cm, 5 µm Zorbax C-18 column. The column was eluted with 90% acetonitrile ($CH_3CN$) for 30 minutes at a flow rate of 0.5 ml/min. The retention times of the sugars in acid hydrolyzate were compared with those of standard sugar samples. The radioactive standard sugars were also used as references to be double sure of the monosaccharides obtained after hydrolysis of glycoproteins.

3.2.19.4 ESTIMATION OF TOTAL CARBOHYDRATE CONTENT

Total carbohydrate content was measured by phenol-sulphuric acid method (64). To a 2 ml aqueous solution (ca 20-75 µg carbohydrate) 50 µl of 90% phenol solution and 5 ml of concentrated $H_2SO_4$ were added, followed by quick stirring on Vortex mixer. The mixture was incubated at room temperature for 30 min and the absorbance of the orange colour was measured at 490 nm against the blank, prepared without sample. Amount of carbohydrate was computed from the standard curve of glucose, prepared under identical conditions.

### 3.2.20 INTER-RELATIONSHIP OF HIGH AND LOW MOLECULAR WEIGHT FORMS OF α-GALACTOSIDASE

To study the inter-relationship of high and low molecular weight forms of α-galactosidase, the two low molecular weight forms α-galactosidase IIa and α-galactosidase IIb (Mr=56,000 and 50,000 daltons), obtained after Sephadex G-150 gel filtration and C M-cellulose chromatography, were stored together as mixture at 4°C in 0.05M acetate buffer, pH 5.2, for 70 days. In the another set of experiment the α-galactosidase IIa and α-galactosidase IIb were stored separately in the similar conditions of temperature and pH. The products were analyzed by Sephadex G-150 gel filtration.

### 3.2.21 IMMOBILIZATION

The α-galactosidase isoenzymes were immobilized in bead-shaped alginate gels.(6,105,167,196). Beads of immobilized enzymes were obtained by preparing a solution of following composition:

| | | |
|---|---|---|
| Sodium alginate | 400 mg | 4.0% (w/v) |
| Egg albumin | 40 mg | 0.4% (w/v) |
| Glutaraldehyde (25%) | 0.1 ml | 0.25% (w/v) |
| Enzyme Soln. | 1.0 ml | |

Total volume made to 10 ml with 0.05 M acetate buffer pH 5.2 (6). The solution was sprayed into a

0.4M $CaCl_2$ solution. After 2 h. of standing the beads of 5 mm average diameter were separated and the enzyme activity was determined by routine method as described below:

A definite number of beads (20 beads) was taken in a test tube. To this 1 ml of 50 mM acetate buffer, pH 5.2 and 0.1 ml (100 μmoles) of substrate were added. The reaction mixture was incubated with continuous shaking at 25°C. After 15 min of incubation the reaction solution was transferred in another test tube to avoid interference of beads in producing colour. The reaction was stopped by adding 1.5 ml of 4% (w/v) sodium carbonate solution. The O.D. was read at 420 nm against a blank solution which was prepared in the same manner using beads without enzyme.

The activity in the control (free enzyme) was used as 100 per cent and the results are expressed as the per cent residual activity immobilized.

3.2.22 DETERMINATION OF DEVELOPMENTAL PATTERN OF α-GALACTOSIDASE ISOENZYMES DURING GERMINATION

In order to study the developmental pattern of α-galactosidases in Cicer-arietinum cotyledons during germination, the Cicer-arietinum seeds were germinated for a period of 7 days as described before. Cotyledons (25-30) were picked up at randomly at various stages of

embryo development and α-galactosidase enzyme was extracted, the high and low molecular forms of α-galactosidase were separated and resolved by Sephadex G-150 gel filtration. All procedures of extraction, isolation, purification and enzyme assay were carried out in the same manner as given before, unless stated otherwise.

3.2.25 SEED GERMINATION IN PRESENCE OF CYCLOHEXIMIDE

For studying the development of α-galactosidase isoenzymes the cycloheximide treatment of seeds was done in the following way: Chickpea seeds were first soaked in distilled water for 12 h to allow the initiation of germination and then transferred to 50 x $10^{-6}$M cycloheximide solution in distilled water for 4-6 h at room temperature. The seeds were removed from the cycloheximide solution, placed in a germinator and allowed to germinate for the indicated periods as described before. Twenty seedlings were picked up at randomly at each stage of germination cotyledons were separated from the plant, the enzymes were extracted and analysed by Sephadex G-150 gel filtration as described in previous sections.

3.2.24 DETERMINATION OF DEVELOPMENTAL PATTERN OF α-GALACTOSIDASE ISOENZYMES DURING SEED MATURATION

To study the developmental pattern of α-galactosidases during maturation, dates of flower opening were recorded and the flowers were tagged. The full anthesis represented '0' day in developmental studies. Pods were removed and seeds taken at different days after flowering. The enzyme was extracted as described before. The changes in the levels of high and low molecular weight forms of α-galactosidase were determined by comparing the Sephadex G-150 gel filtration profiles at each stage of flowering.

3.2.25 STUDY OF ISOENZYMIC PATTERN OF α-GALACTOSIDASE ISOENZYMES IN DIFFERENT VARIETIES OF CICER-ARIETINUM SEEDS

The isoenzymic patterns of α-galactosidases in different varieties of Cicer-arietinum seeds such as of white variety (Kabli chickpea seeds), and of black varieties ($H_{208}$ and $C_{235}$), were investigated in the following manner: Varieties of chickpeas that can be distinguished by their superficial parameters such as-colour, shape and size were separated. The enzyme was extracted in the normal way and subjected to pH precipitation and ammonium sulphate fractionation followed by dialysis as described before. The polyacrylamide gel electrophoresis of the dialyzed samples was carried out

on 7.5% gels according to the procedure described before and the patterns of α-galactosidase isoenzymes in the different varieties were compared.

The isoenzymic patterns of black variety chickpea seeds and white variety (Kabli chickpeas) seeds were studied further by comparing the Sephadex G-150 gel filtration profiles at different stages of seed germination and seed maturation.

## 4.0 RESULTS

### 4.1 α-GALACTOSIDASE ACTIVITY AS A FUNCTION OF GERMINATION PERIOD

In order to obtain chickpea (Cicer-arietinum) cotyledons containing high level of α-galactosidase activity, germinating cotyledons of different ages were analysed for total α-galactosidase activity. The results given in Fig.2 show that dormant seeds contain very low activity of α-galactosidase compared to the germinating seeds. However, after 24 h of imbibition seeds in water at 25°C the enzyme activity rose sharply reaching a maximum level on the 6th day of germination. Thereafter, the enzyme activity declined rapidly. For instance, on the 7th day of germination only one-half of the maximum α-galactosidase activity was found. Thus, 6-days old germinating chickpea cotyledons were used for the purification of various isoenzymes of α-galactosidase.

### 4.2 SEPARATION AND PURIFICATION OF MULTIMOLECULAR FORMS OF α-GALACTOSIDASE FROM CHICKPEAS

Fig.3 shows the stepwise flow diagram for the isolation of α-galactosidase isoenzymes. The purification data of various forms of α-galactosidase from the six-days old germinating chickpea cotyledons are summarized in the Table III. The purification scheme

FIG.2. ACTIVITY OF α-GALACTOSIDASE OF CHICKPEA COTYLEDONS AS A FUNCTION OF GERMINATION PERIOD.

Seeds were germinated in dark at 25°C for the indicated periods. Twenty seeds were excised, enzyme extract was prepared, protein and enzyme activity were determined as described under 'Experimental Procedures'. One enzyme unit represents the amount of protein in mg which liberated 1 μmole p-nitrophenol from p-nitrophenyl-α-D-galactopyranoside under the assay conditions used. The maximum activity was observed between 6th and 7th day of germination.

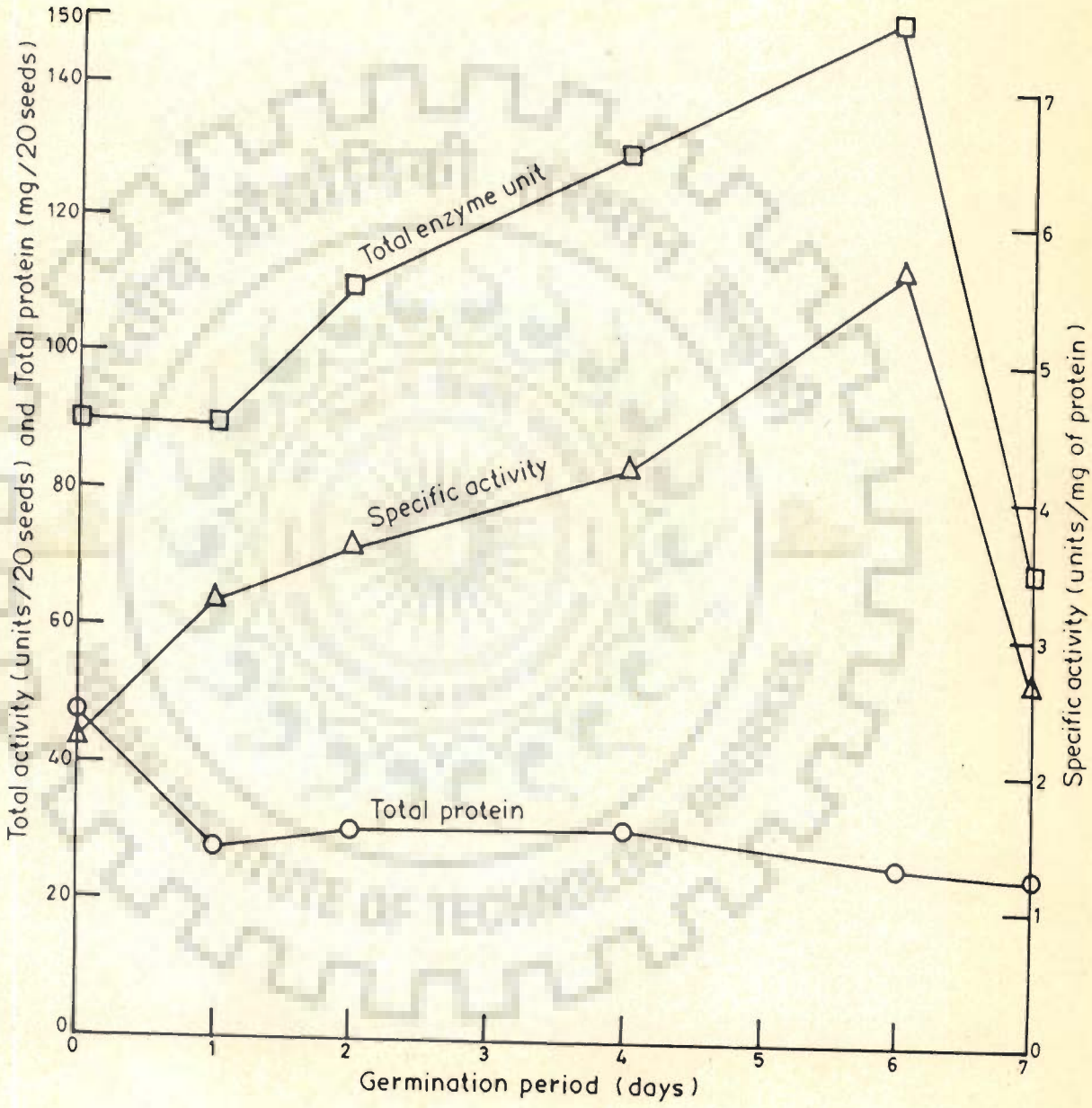

Total activity ( units / 20 seeds ) and Total protein (mg / 20 seeds )
Specific activity (units/mg of protein )
Germination period ( days )
Total enzyme unit
Specific activity
Total protein
150
140
120
100
80
60
40
20
0
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7

FIG. 5. FLOW DIAGRAM FOR THE SEPARATION AND PURIFICATION OF α-GALACTOSIDASE ISOENZYMES FROM CHICKPEA COTYLEDONS.

Two hundred grams chickpea cotyledons (fresh weight) were used to prepare the crude homogenate.

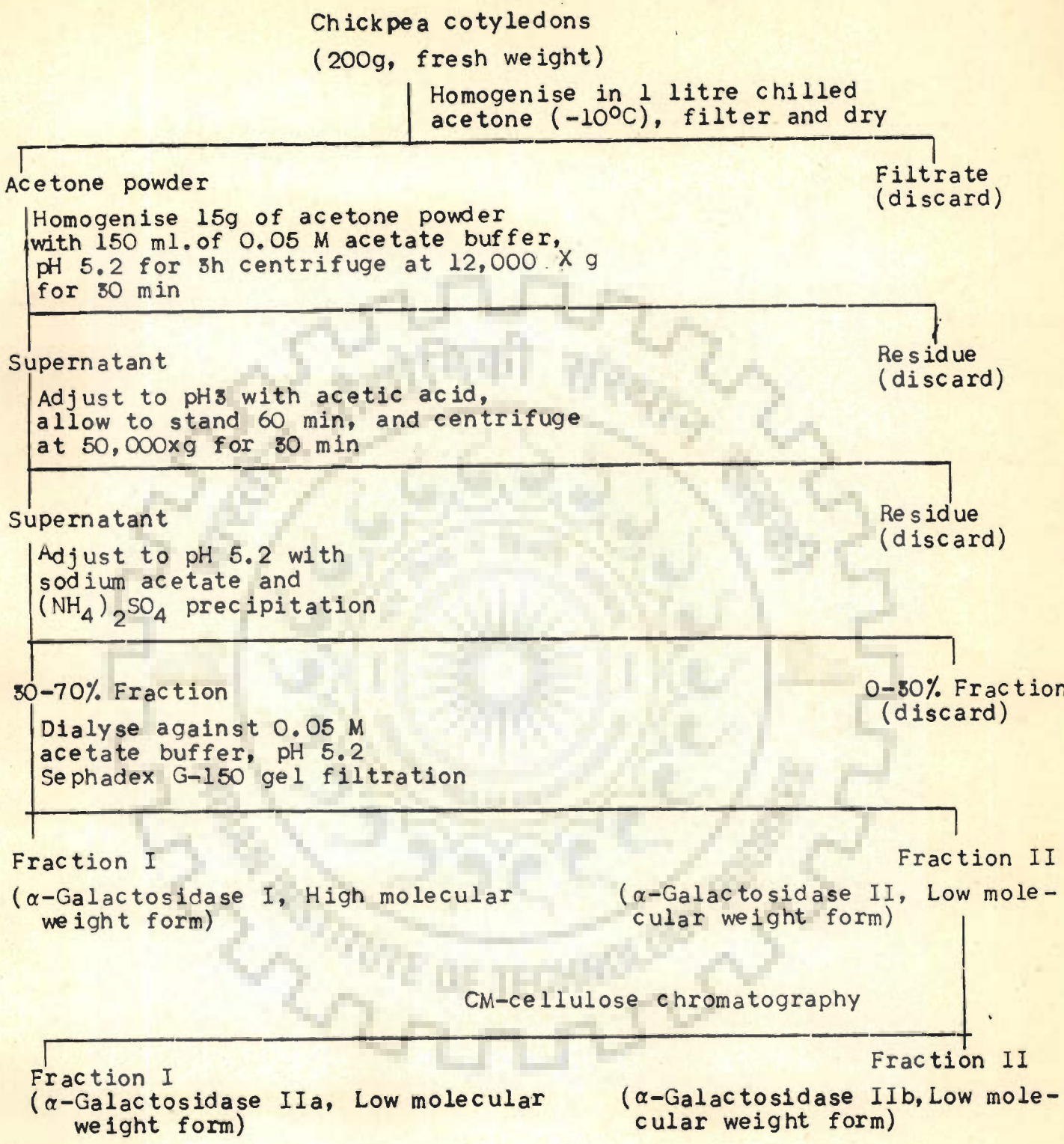



Fig.3. Flow diagram for the separation and purification of α-galactosidase isoenzymes from chickpea cotyledons.

TABLE III PURIFICATION OF MULTIPLE FORMS OF α-GALACTOSIDASE FROM CICER-ARIETINUM SEEDS

| Purification step | Total protein (mg) | Total activity (units) | Specific activity ( units/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| 1. Crude extract | 694 | 1458 | 2.02 | 100 | 0.0 |
| 2. Acetone powder extract | 540 | 1250 | 2.31 | 85.7 | 1.1 |
| 3. pH precipitation | 336 | 1244 | 3.7 | 85.3 | 1.8 |
| 4. Ammonium sulphate precipitation | | | | | |
| (i) 0.3 saturation | 155 | 51 | 0.322 | 3.5 | nil |
| (ii) 0.7 saturation | 147.6 | 840 | 5.69 | 57.6 | 2.8 |
| 5. Sephadex G-150 gel filtration | | | | | |
| Peak I (α-galactosidase I ) | 3.8 | 26 | 7.01 | 1.8 | 3.4 |
| Peak II (α-galactosidase II) | 17.52 | 380 | 21.69 | 26.0 | 10.7 |
| 6. CM-cellulose chromatography of α-galactosidase II | | | | | |
| Peak I (α-galactosidase IIa) | 2.93 | 264 | 90.09 | 18.0 | 44.6 |
| Peak II (α-galactosidase IIb) | 1.28 | 96 | 75.18 | 6.6 | 37.2 |

involved six main steps in the following sequence: (i) acetone powder preparation (ii) extraction of α-galactosidase enzyme (iii) pH-precipitation (iv) ammonium sulphate fractionation (v) Sephadex G-150 gel filtration and (vi) CM-cellulose chromatography.

The acetone powder preparation was an essential preliminary treatment since besides yielding 1.1 fold purification it removed lipids, pigments and other such materials which usually interfere in the isolation of enzymes. Furthermore, the acetone powder can be stored easily for several months at $0^{o}C$ in anhydrous condition without any loss of enzyme activity enabling us to use the same sample through out the studies and thereby minimizing the possibility of properties variation due to sample differences.

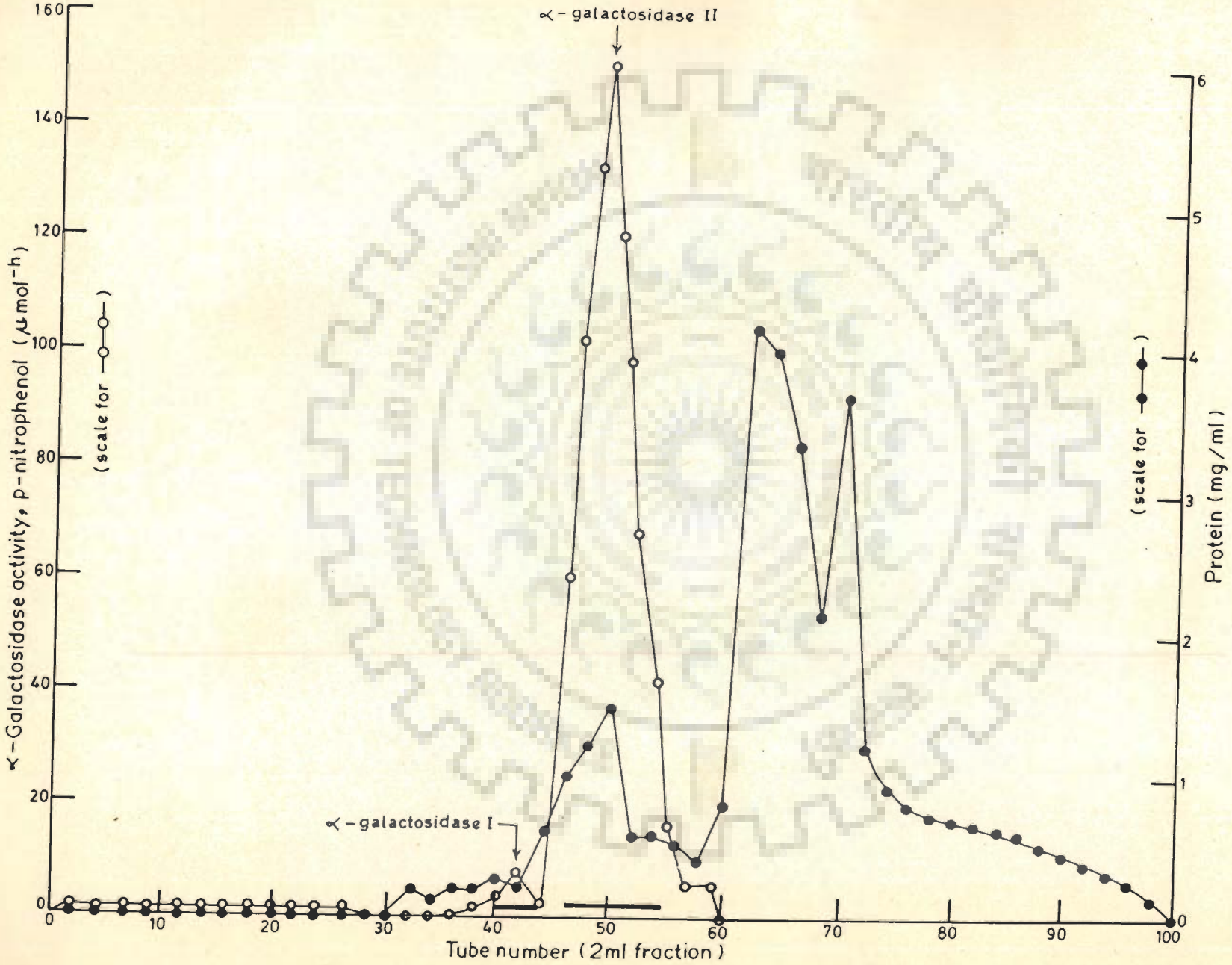
The next step involved the selective pH-precipitation. It was found that when cell free crude homogenate prepared from acetone powder, was brought to pH 3.0 with 1.0 M acetic acid, a large amount of protein was precipitated which was devoid of α-galactosidase activity. In contrast, the soluble fraction was found to contain almost total enzyme activity with 1.8 fold increase in the specific activity.

The $(NH_4)_2SO_4$ fraction precipitating between 0.3 and 0.7 saturation contained most of the enzyme. Other fractions showed only little α-galactosidase activity and were rejected. The 0.3 - 0.7 fraction was dissolved in 0.05 M acetate buffer, pH 5.2, and dialysed against the same buffer in a Visking cellophane tubing to remove compounds smaller than 15,000 molecular weight. At this stage the purification and the yield of α-galactosidase were 2.8 fold and 57.6% , respectively.

This fraction was subjected to the gel filtration on Sephadex G-150 gel column. Fig.4 shows the elution profile of α-galactosidase enzyme from the Sephadex G-150 column. Two major peaks, peak I and peak II, containing α-galactosidase activity were obtained. Peak I which was eluted first contained the high molecular weight α-galactosidase species (α-galactosidase I) and the peak II which eluted later contained the low molecular α-galactosidase species (α-galactosidase II). The purification of α-galactosidase I and α-galactosidase II was 3.4 and 10.7 folds, with per cent yields of 1.8 and 26.0, respectively. Since α-galactosidase I accounted for only a relatively small amount (about 7% ) of the total α-galactosidase activity, it was not processed further.

FIG.4. SEPARATION OF HIGH MOLECULAR WEIGHT AND LOW MOLECULAR WEIGHT FORMS OF α-GALACTOSIDASE BY SEPHADEX G-150 GEL FILTRATION.

0.5-0.7 ammonimum sulphate fraction was dissolved in a minimal volume of 0.05M sodium acetate buffer, pH 5.2 and dialyzed overnight against the same buffer. A 5 ml. sample (ca 125 mg protein) was loaded on the column (2x80cm), previously equilibrated with the elution buffer (50 mM sodium acetate buffer, pH 5.2). The absorbed protein was eluted with the same buffer at a flow rate of 0.1 ml/ min. Two ml fractions were collected and aliquots (0.1 ml) from every other fractions were analysed for protein and α-galactosidase activity as described under 'Experimental Procedure'. The fractions indicated by bar (—) were pooled and used for further studies. o——o, enzyme activity ; •——•, protein content.


∝-galactosidase II
∝-galactosidase I
∝-Galactosidase activity, p-nitrophenol (μmol⁻¹h)
(scale for —○—)
Protein (mg/ml)
(scale for —●—)
Tube number (2ml fraction)
0
20
40
60
80
100
120
140
160
1
2
3
4
5
6
10
30
50
70
90

## 4.3 PURIFICATION OF α-GALACTOSIDASE-IIa AND α-GALACTOSIDASE-IIb

The α-galactosidase II peak from the Sephadex G-150 column which accounted for over 93% of the total α-galactosidase activity in the sample, was subjected to DEAE-cellulose and CM-cellulose column chromatography for further purification and separation of different isoenzymes. Fig.5 shows the elution profile of α-galactosidase II from the DEAE-cellulose column. It was found that almost all the α-galactosidase activity was lost when this column was run at 7.2 pH using a step wise gradient of 25, 50, 100, 200, 300, 400 and 500 mM Nacl. These results indicated that the enzyme was extremely unstable under alkaline conditions. Thus, it was decided to use CM-cellulose chromatography in acidic medium, pH 5.2.The elution profile is shown in Fig.6. Two distinct peaks showing α-galactosidase activity were eluted with 240 mM and 345 mM NaCl gradient. These fractions were designated as α-galactosidase IIa and α-galactosidase IIb, respectively. The purification of α-galactosidase IIa and α-galactosidase IIb was 44.6 and 57.2 folds with per cent yields of 18.0 and 6.6, respectively.

## 4.4 HOMOGENEITY

The protein fractions containing α-galactosidase activity (α-galactosidase IIa and α-galactosidase IIb)

FIG. 5. DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF α-GALACTOSIDASE II.

The α-galactosidase II fraction obtained after the Sephadex G-150 gel filtration containing 15 mg of protein was chromatographed on DEAE-cellulose column (2x25 cm) by stepwise elution with 0.05 M, pH 7.2, phasphate buffer containing increasing sodium chloride concentration (25 mM, 50 mM, 100 mM, 200 mM, 300 mM, 400 mM and 500 mM). One ml fractions were collected at a flow rate of 0.2 ml/min and from every other fraction, aliquots (0.2 ml) were tested for protein content and α-galactosidase activity as described under 'Experimental Procedure' o——o, enzyme activity ; ●——● protein content

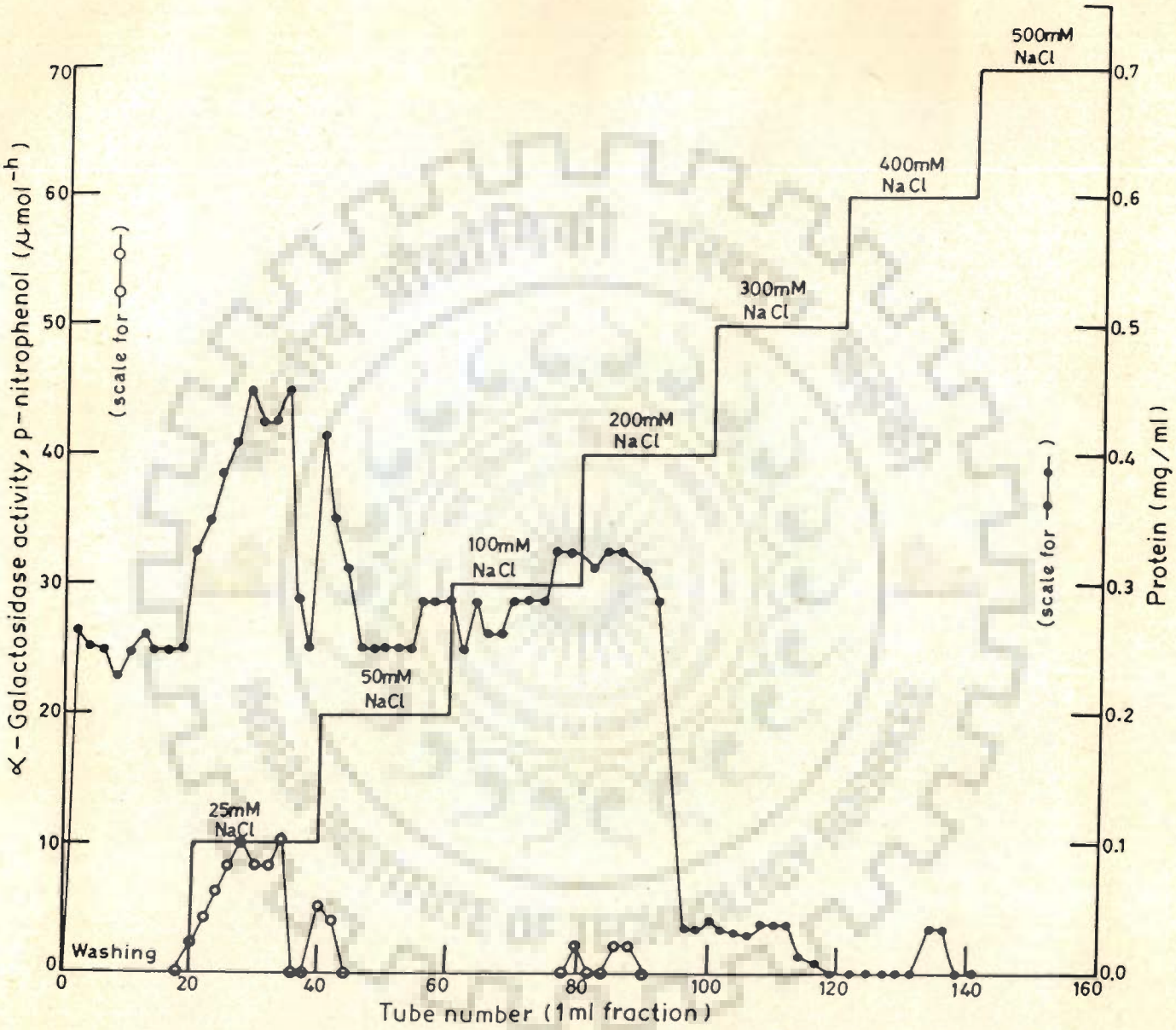
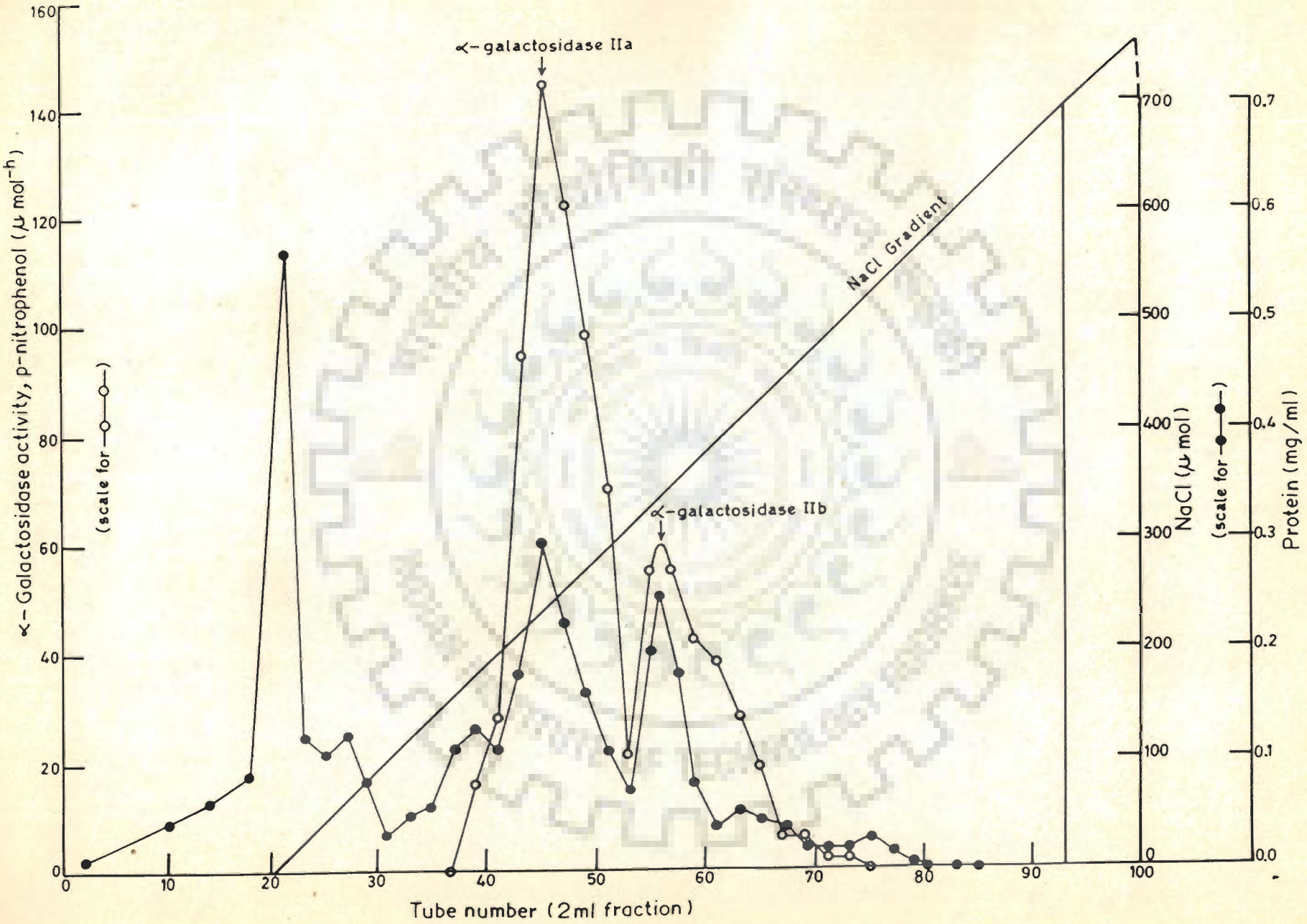

500mM NaCl
400mM NaCl
300mM NaCl
200mM NaCl
100mM NaCl
50mM NaCl
25mM NaCl
Washing
(scale for —o—o—)
(scale for —•—•—)
∝ - Galactosidase activity, p-nitrophenol (μmol⁻ʰ)
Protein (mg/ml)
Tube number (1ml fraction)
0
10
20
30
40
50
60
70
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
20
40
60
80
100
120
140
160

FIG.6. CM-CELLULOSE COLUMN CHROMATOGRAPHY OF α-GALACTOSIDASE II

The α-galactosidase II fractions obtained after Sephadex G-150 gel filtration (pooled fractions indicated by bar '——') containing 15 mg of protein was chromatographed on CM-cellulose column (2x25 cm) by a linear gradient of sodium chloride (0-700 mM) in 0.05M, sodium acetate buffer pH 5.2 as described in the text. A flow rate of 0.2 ml/min was used and fractions (1 ml each) were collected. Aliquotes (0.2 ml) from every other fraction analyzed for α-galactosidase activity and protein content. The fractions indicated by the bar (——) were pooled and used for further studies. Peak I (fraction no.45-49) and Peak II (Fraction No.55-57) were found pure by polyacrylamide gel electrophoresis.


α-galactosidase IIa
α-galactosidase IIb
NaCl Gradient
α-Galactosidase activity, p-nitrophenol (μ mol⁻ʰ)
(scale for —o—o—)
NaCl (μ mol)
(scale for —●—●—)
Protein (mg/ml)
Tube number (2ml fraction)
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
160
0
100
200
300
400
500
600
700
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7

were examined by polyacrylamide gel electrophoresis at pH 6, pH 7.5 and pH 8 single band was obtained in each case indicating that these enzyme preparations were homogeneous (Fig.7). The electrophoretic mobilities of IIa and IIb enzymes were found to be 0.24 and 0.32 respectively.

## 4.5 PROPERTIES OF THE α-GALACTOSIDASES

### 4.5.1 MOLECULAR WEIGHTS OF α-GALACTOSIDASE ISOENZYMES

The molecular weights of various forms of α-galactosidase were determined by Sephadex gel filtration using the following standard proteins: cytochrome C (Mr = 12,400), bovine serum albumin (Mr = 66,500) and alkaline phosphatase (Mr = 100,000). The elution pattern and calibration curve of standard proteins are shown in Fig.8. The molecular weight of α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb were found to be 149,600, 56,000 and 50,000 daltons, respectively.

### 4.5.2 BEHAVIOUR ON SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzymes and standard proteins was performed in presence of the β-mercaptoethanol. A plot of electrophoretic mobilities vs. $\log_{10}$ Mr is shown in Fig.9, α-galactosidase IIa produced three protein bands corresponding to Mr=56,000, 22,000, and 16,000 daltons, respectively. Their electrophoretic

FIG.7. POLYACRYLAMIDE GEL ELECTROPHORESIS OF α-GALACTOSIDASE ISOENZYMES.

The electrophoresis was carried out with 7.5% gels by the method described in 'Experimental Procedures', Samples were Sephadex gel filtration peak II (S II ca 150 µg protein); Sephadex gel filtration peak I (S I, ca 150 µg protein); CM-cellulose peak I (CM IIa ca 80 µg protein) and CM-cellulose peak II (CM IIb, ca 80 µg protein). Direction of electrophoresis was from top to bottom.

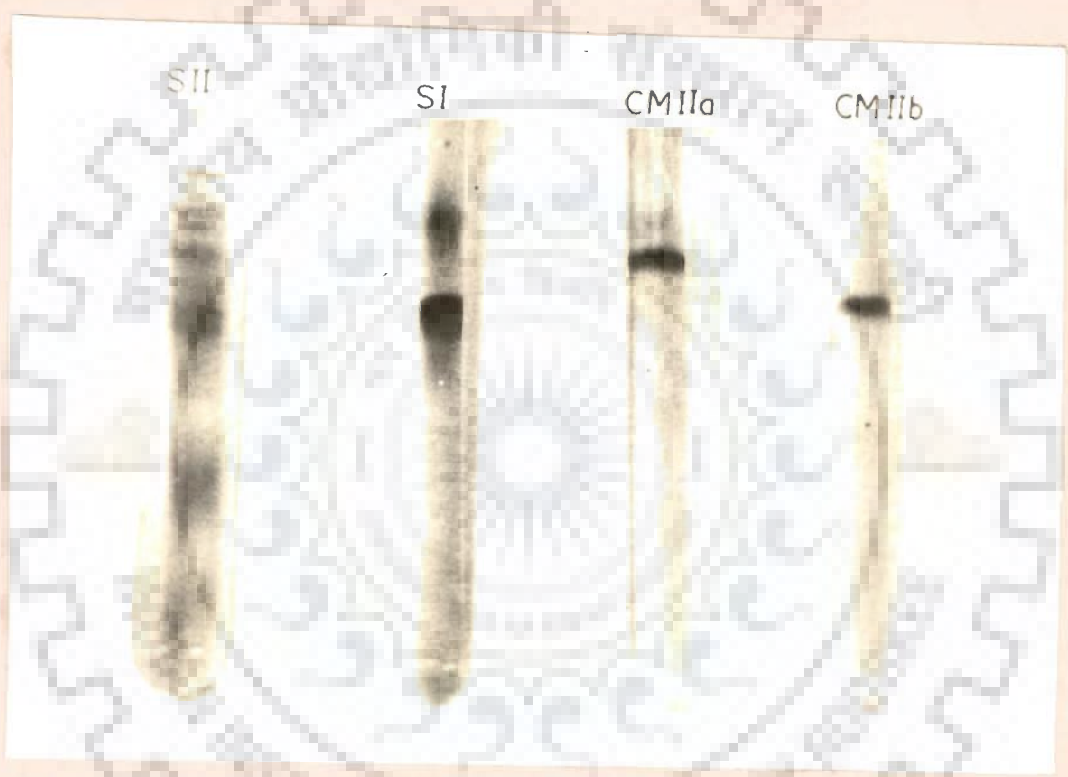
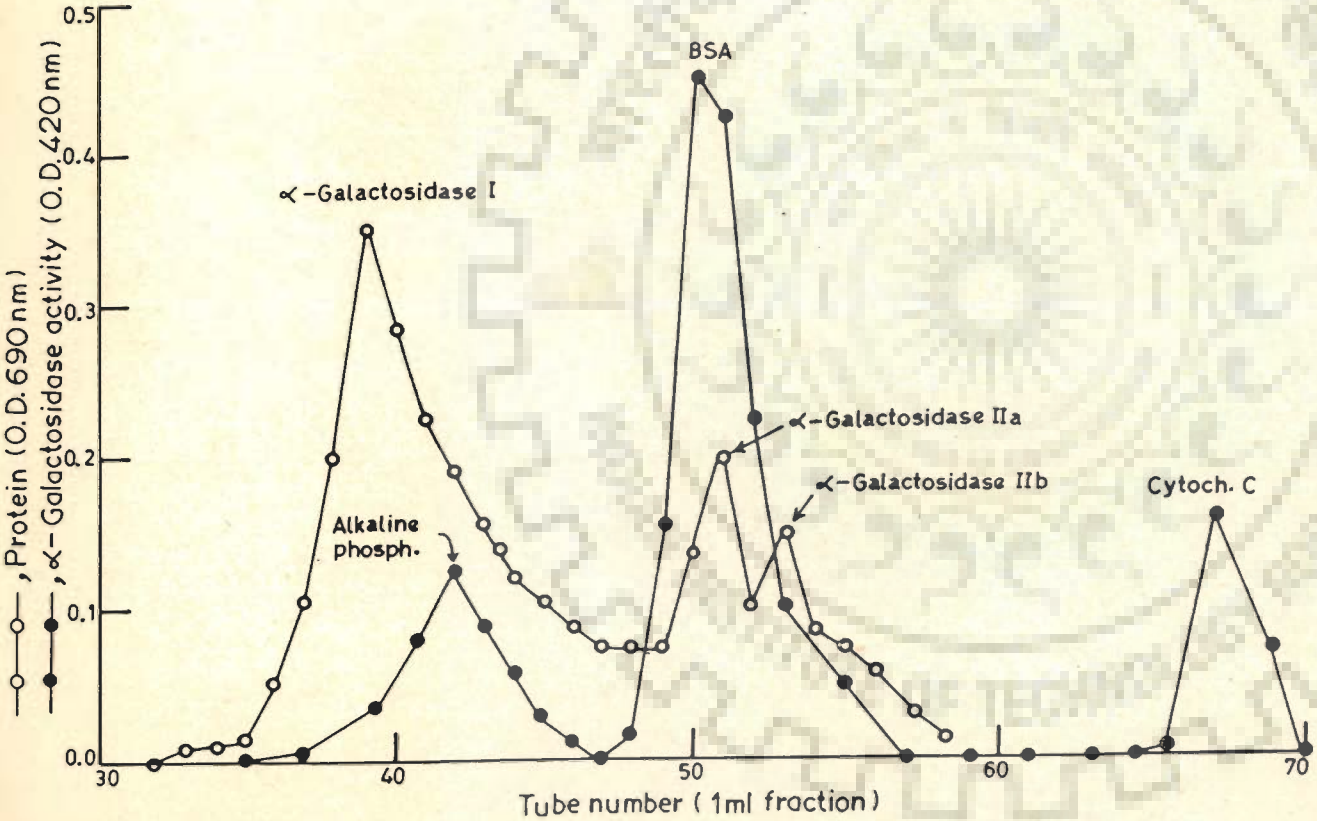
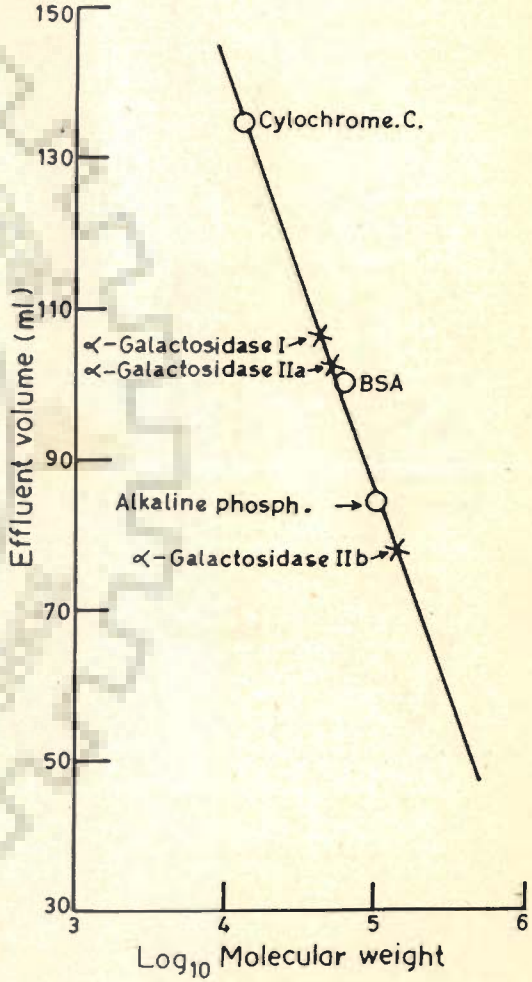

SII
SI
CMIIa
CMIIb

FIG.8. ESTIMATION OF MOLECULAR WEIGHT OF CHICKPEA α-GALACTOSIDASE ISOENZYMES BY SEPHADEX GEL FILTRATION.

A standard protein mixture (cytochrome C Mr = 12,400 daltons; BSA, bovine serum albumin Mr = 66,500 daltons and alkaline phosphatase Mr = 100,000 daltons; 5 mg each) was eluted from Sephadex G-150 column (1x80 cm) with 0.05 M sodium acetate buffer pH 5.2, at a flow rate of 0.1 ml/min. Fractions of 2 ml were collected and analysed for protein contents. A standard curve was prepared by plotting the elution volume versus $\log_{10}$ molecular weight of standard proteins. Elution experiments with α-galactosidase isoenzymes were performed using the same column under identical experimental conditions. Molecular weights of α-galactosidase I, IIa and IIb were computed from standard curve. The calculations were based on average of 3 independent runs.


0.5
0.4
0.3
0.2
0.1
0.0
, Protein (O.D. 690 nm)
, ∝-Galactosidase activity (O.D. 420 nm)
∝-Galactosidase I
BSA
∝-Galactosidase IIa
∝-Galactosidase IIb
Alkaline phosph.
Cytoch. C
30
40
50
60
70
Tube number ( 1ml fraction )

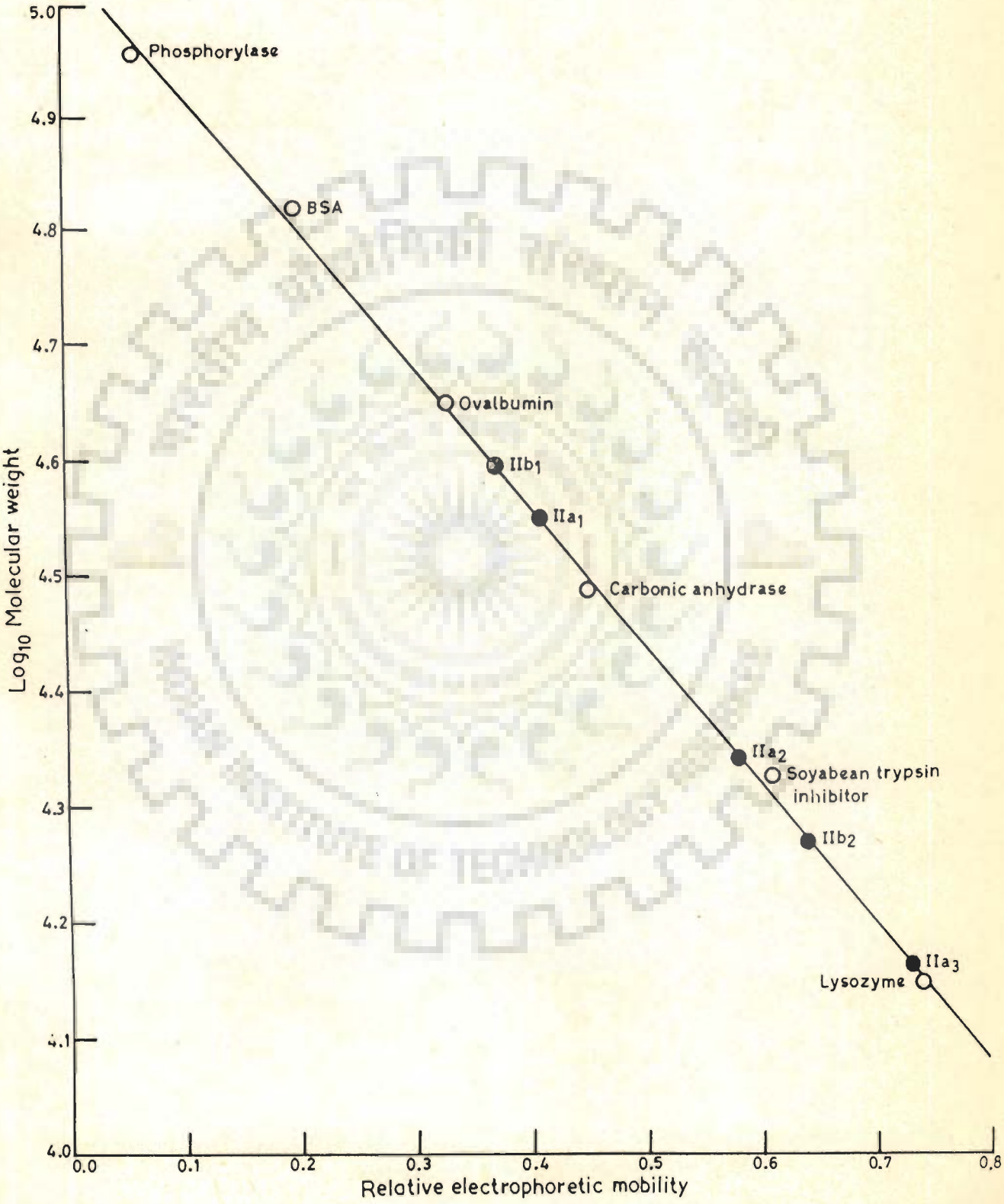
(A)


150
130
110
90
70
50
30
Effluent volume (ml)
Cylochrome.C.
∝-Galactosidase I
∝-Galactosidase IIa
BSA
Alkaline phosph.
∝-Galactosidase IIb
3
4
5
6
Log10 Molecular weight

(B)

FIG.9. ESTIMATION OF MOLECULAR WEIGHT OF CHICKPEA α-GALACTOSIDASE ISOENZYMES BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Electrophoresis was performed on 10% polyacrylamide gel under fully dissociating conditions as described under 'Experimental Procedures'. Mobilities were determined relative to the migration of bromophenol blue, the tracking dye. The molecular weights of the purified enzyme subunits ($IIa_1$, $IIa_2$, $IIa_3$, $IIb_1$ and $IIb_2$) were computed from the calibration plot of $Log_{10}$ molecular weight versus relative mobility of standard proteins (phosphorylase b, Mr = 92.5K ; bovine serum albumin, Mr = 66.2K; ovalbumin, Mr=45.0K ; carbonic anhydrase, Mr = 31.0K ; soybean trypsim inhibitor, Mr = 21.5K and lysozyme, Mr = 14.4K ). The calculations were based on average of 3 independent electrophoretic runs.


5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
Log10 Molecular weight
Phosphorylase
BSA
Ovalbumin
IIb1
IIa1
Carbonic anhydrase
IIa2
Soyabean trypsin inhibitor
IIb2
IIa3
Lysozyme
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Relative electrophoretic mobility

mobilities were 0.41, 0.58 and 0.73, relative to bromophenol blue in 0.05 M phosphate buffer, pH 7.5. The α-galactosidase IIb was dissociated in two protein bands of Mr = 40,000 and 18,000 daltons with the electrophoretic mobilities relative to bromophenol blue of 0.37 and 0.64, respectively (Fig.10). Since, the electrophoresis was performed under completely dissociated conditions, the α-galactosidase IIa and IIb appear to have three and two subunits, respectively. However, at the moment presence of more than one polypeptide chains of exactly the same size in each protein band can not completely ruled out.

### 4.5.3 pH-PROFILES

The effect of pH on the activity of different molecular forms of α-galactosidase was studied by using p-nitrophenyl α-D-galactopyranoside as substrate. The pH-activity plots are shown in Fig.11. The pH activity curves for α-galactosidase I, IIa and IIb are sharply peaked at pH 5.2, 5 to 5.2, 5.5, respectively.

### 4.5.4 SUBSTRATE SPECIFICITY OF DIFFERENT α-GALACTOSIDASE ISOENZYMES

The substrate specificity of α-galactosidase isoenzymes was compared using various substrates. Data given in Table IV shows that beside p-nitrophenyl-α-D-

FIG. 10. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS.

The electrophoresis was carried out with 10% gels, samples were prepared and handled as described under 'Experimental Procedures'. Samples and amounts loaded were : std., standard (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme; 10 µg each ); CM IIa, CM cellulose peak I. (80 µg-protein); CM IIb, CM cellulose peak II (80 µg protein).

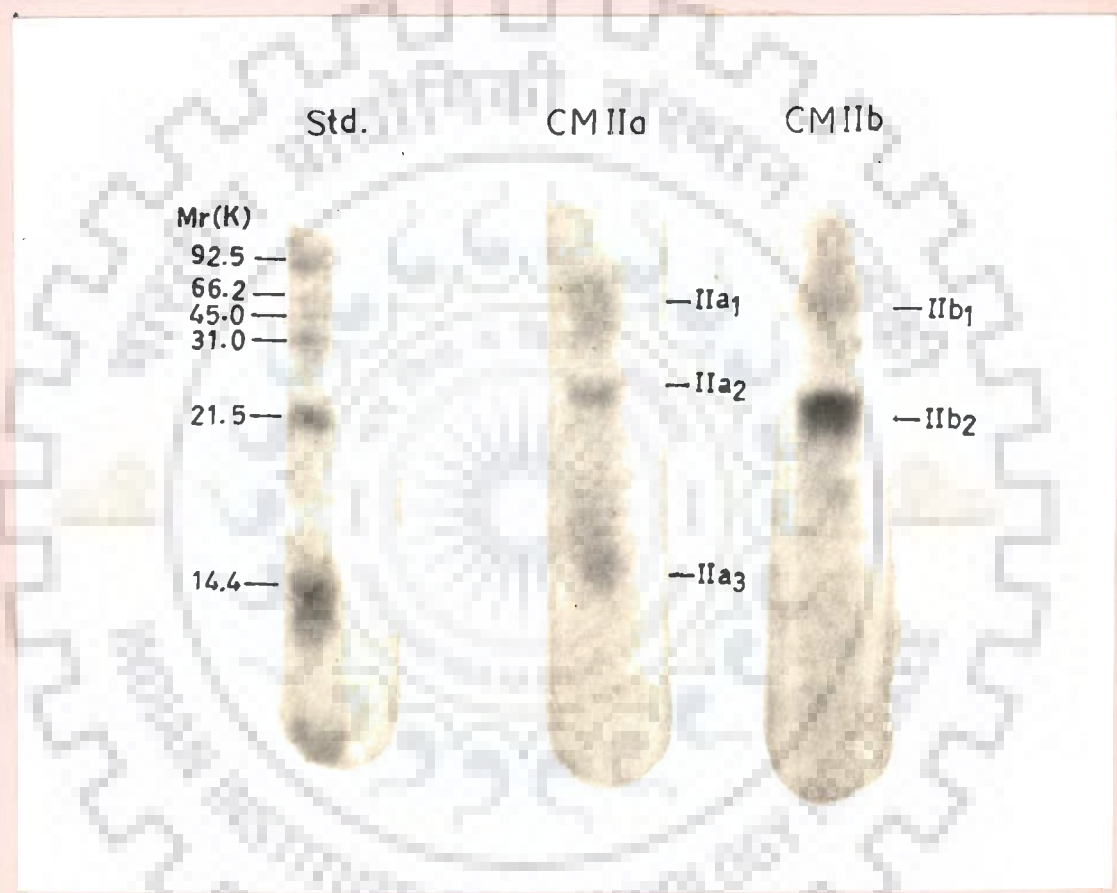

Std.
CM IIa
CM IIb
Mr(K)
92.5
66.2
45.0
31.0
21.5
14.4
—IIa1
—IIa2
—IIa3
—IIb1
—IIb2

FIG.11. EFFECT OF pH ON FREE AND IMMOBILIZED CHICKPEA α-GALACTOSIDASE ISOENZYMES

The enzyme activity was measured in the standard assay system except that the pH of the buffer varied from 3.0 to 8.0. The pH of the buffer was adjusted by sodium hydroxide solution.

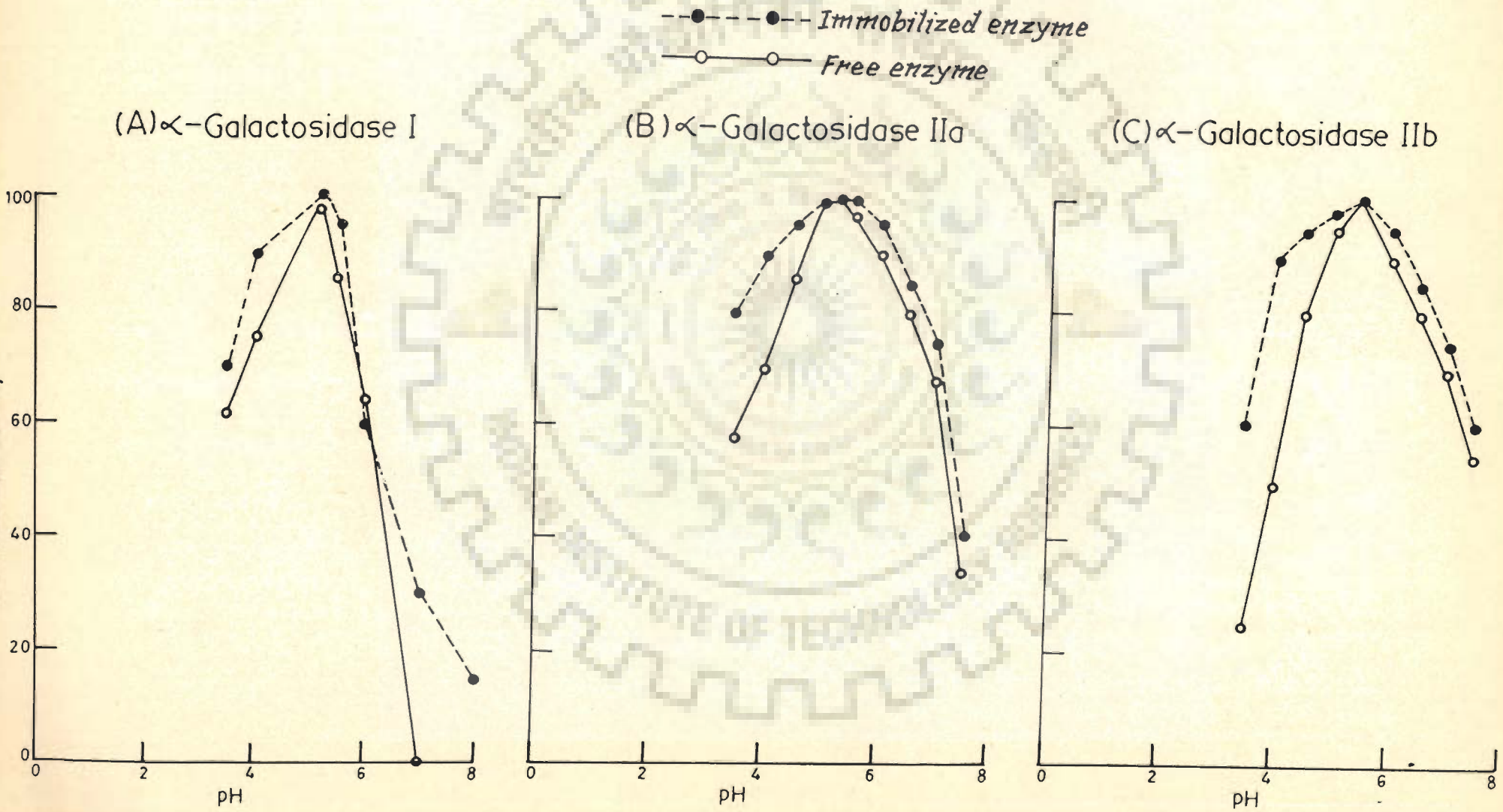

Immobilized enzyme
Free enzyme
(A) ∝-Galactosidase I
(B) ∝-Galactosidase IIa
(C) ∝-Galactosidase IIb
100
80
60
40
20
0
0
2
4
6
8
pH

TABLE IV SUBTRATE SPECIFICITY

| Substrate | α-galactosidase isoenzymes | | | | | |
|---|---|---|---|---|---|---|
| | I | | IIa | | IIb | |
| p-nitro-phenyl-β-galactoside | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) |
| p-N-P-β-galactoside[a] | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| p-N-P-α-glucoside | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| p-N-P-β-glucoside | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| p-N-p-α-mannoside | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| p-N-P-β-Mannoside | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| p-N-P-β-N-acelyl-glucosaminidase | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| p-N-P-β-Xyloside | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Melibiose(Gal-Glu) | 1.6 | 2.5 | 2.8 | 0.9 | 3.0 | 9.5 |
| Raffinose (Gal-Glu-Fruc) | 4.2 | 23.2 | 6.9 | 5.8 | 4.5 | 2.0 |
| Stachyose (Gal-Gal-Glu-Fruc) | 6.9 | 11.4 | 8.1 | 2.1 | 2.4 | 1.1 |
| p-N-P-α-galactopyrano-side | 0.35 | 5.263 | 0.13 | 2.0 | 0.27 | 0.99 |

[a] p-N-P = para nitrophenyl

galactopyronoside, the enzymes are also found active towards melibiose, raffinose and stachyoses. All the substrates contain α-D-galactosyl residue as the non-reducing sugar terminal indicating that the enzymes are highly specific for both the monosaccharide moiety and the anomeric linkage on $C_1$ of the monosaccharides. Other substrates containing either β-glycosidic linkage or glycosides other than galactopyronoside were not hydrolyzed. A comparision of the $K_m$ and $V_{max}$ values (using $V_{max}/K_m$ as the basis of comparision) shows that for α-galactosidase I p-nitrophenyl-α-D-galactopyronoside was the best substrate followed by raffinose, stachyose and melibiose; the last two being comparable. For α-galactosidase IIa p-nitrophenyl α-D-galactopyranoside, appear to be the best substrate followed by raffinose, melibiose and stachyose. For α-galactosidase IIb melibiose and p-nitrophenyl α-D-galactopyranoside appear to be much better substrate than raffinose and stachyose. The order of substrate affinity ($1/K_m$) for α-galactosidase I, IIa and IIb is also significantly different.

From these results it seems possible that α-galactosidase isoenzymes can be distinguished on the basis of substrate specificity.

### 4.5.5 INHIBITION OF α-GALACTOSIDASE ISOENZYMES BY SUGARS AND RELATED COMPOUNDS

In order to see the effect of various sugars on different α-galactosidase isoenzymes, enzyme assays were carried out under identical conditions in the presence of different monosoccharides and related compounts (Table V). Of the various sugars used D(+) xylose, L(+) arabinose, D(+) galactose, arabitol and myoinositol were found to inhibit all the forms of α-galactosidase. However, degree of inhibition by different sugars was somewhat different. For instance, at 50 mM concentration, xylose inhibited α-galactosidase I, IIa and IIb by 70.5, 66 and 49%, respectively. Galactose at 5 mM concentration yielded a comparable inhibitory effect on different α-galactosidase isoenzymes. Arabinose, myoinositol and arabitol require much higher concentration for producing comparable inhibitory effects. D-Mannose, D-glucose, fructose, xylitol, galactitol, sorbitol and (+) inositol and myoinositol-2-phosphate do not produce any detectable inhibitory effect on the activity of α-galactosidase. These results once again show the glycon specificity of α-galactosidase isoenzymes. In some cases the degree of inhibition of different isoenzymes is markedly different which provides a method of distinguishing between various forms of isoenzymes.

TABLE V INHIBITION OF α-GALACTOSIDASE ISOENZYMES BY SUGARS AND RELATED COMPOUNDS

| Inhibitor | Conc. (mM) | Per cent inhibition α-Galactosidase isoenzymes | | |
|---|---|---|---|---|
| | | I | IIa | IIb |
| D(+) Xylose | 50 | 70.5 | 66 | 49 |
| L(+) Arabinose | 100 | 50.3 | 39 | 25 |
| D(+) Galactose | 5 | 74.0 | 58 | 38 |
| D(+) Mannose | 200 | 0.0 | 0.0 | 0.0 |
| D(+) Clucose | 200 | 0.0 | 0.0 | 0.0 |
| Fructose | 200 | 0.0 | 0.0 | 0.0 |
| Xylitol | 200 | 0.0 | 0.0 | 0.0 |
| Arabitol | 200 | 70.8 | 53 | 41 |
| Galactitol | 200 | 0.0 | 0.0 | 0.0 |
| Sorbitol | 200 | 0.0 | 0.0 | 0.0 |
| (+) Inositol | 200 | 0.0 | 0.0 | 0.0 |
| Myoinositol-2-Phosphate | 200 | 0.0 | 0.0 | 0.0 |
| Myoinositol | 50 | 61.2 | 59.8 | 52.0 |

### 4.5.6 EFFECT OF METAL IONS

Table VI shows the effect of some common metal ions. It can be seen from the results that silver and mercury ions are very strong inhibitors of different α-galactosidase isoenzymes. For instance, silver ions at 2 μmol concentration inhibit α-galactosidase I, IIa and IIb by 60, 81 and 65% , respectively. Similarly mercury at 2 μmol concentration produced 30% , 65% and 55% inhibition of α-galactosidase I, IIa and IIb, respectively. All other metal ions including cadmium and copper do not have any significant effect on the activity of α-galactosidase isoenzymes. The degree of inhibition of metal ions of different α-galactosidase isoenzymes varies quite significantly,e.g., at 1 μmol concentration of silver only one fourth of the activity of α-galactosidase I was inhibited whereas 50% of α-galactosidase IIa and nearly 40% of α-galactosidase IIb was inhibited. The variation of inhibition by metal ions, once again, provide a method of distinguishing α-galactosidase isoenzymes. In general, silver and mercury ions inhibit α-galactosidase IIa form more than that of α-galactosidase I or IIb. The physiological significance of metal ion inhibition is not known. However, these results suggest that sulfahydryl groups may be involved in the active site of the enzyme, since, both mercury and silver ions inhibit all the enzymes

TABLE VI EFFECT OF METAL IONS ON THE ENZYMATIC ACTIVITY OF α-GALACTOSIDASE ISOENZYMES

| Metal ions | Conc. (μmol) | Per cent Inhibition | | |
|---|---|---|---|---|
| | | α-galactosidase isoenzymes | | |
| | | I | IIa | IIb |
| $Ag^{+}$ | 1 | 25 | 50 | 39 |
| | 2 | 60 | 81 | 65 |
| | 4 | 75 | 95 | 75 |
| $Hg^{2+}$ | 1 | 15 | 40.1 | 31.6 |
| | 2 | 30 | 65.0 | 55.2 |
| | 4 | 42 | 74.6 | 60.3 |
| $Cu^{2+}$ | 100 | 2 | 1 | 0.0 |
| | 200 | 4 | 1.5 | 0.0 |
| | 400 | 7 | 2.0 | 0.0 |
| $Mn^{2+}$ | 100 | -4.4 | -6.2 | -3.8 |
| | 200 | -8.0 | -8.6 | -6.2 |
| | 400 | -12.0 | -12.0 | -9.2 |
| $Zn^{2+}$ | 100 | 0.0 | 0.0 | 0.0 |
| | 200 | 0.0 | 0.0 | 0.0 |
| | 400 | 0.0 | 0.0 | 0.0 |
| $Mg^{2+}$ | 1000 | 0.0 | 0.0 | 0.0 |
| | 2000 | 0.0 | 0.0 | 0.0 |
| | 3000 | 0.0 | 0.0 | 0.0 |
| $Pb^{2+}$ | 500 | 3 | 0.0 | 0.0 |
| $Cd^{2+}$ | 500 | 6.3 | 4.2 | 2.8 |
| $Ca^{2+}$ | 1000 | 0.0 | 0.0 | 0.0 |
| $K^{+}$ | 1000 | 0.0 | 0.0 | 0.0 |

competitively (Table VII). A comparision of $K_i$ values shows that α-galactosidase I has the lowest affinity for silver and mercury ions.

4.5.7 THERMAL STABILITY

The optimum temperature of the α-galactosidases was determined by incubating the different isoenzymes to various temperatures for a period of 30 minutes and then assaying the residual activity as described in section '2.4'. The results are shown in Fig.12. From these results it is apparent that α-galactosidase I, IIa and IIb show 100% stability only upto 25°C - 30°C , 25°C and 25°C, respectively. After that there is a rapid decline in the enzyme activity.

When the α-galactosidase isoenzymes were exposed at 50°C for different time periods, they showed a rapid decline in the enzymatic activity. Fig.13 showed that only 45% , 30% and 50% of the maximum activities have been retained by α-galactosidase I, IIa and IIb just after 5 minutes and while 30 min exposure rendered the enzymes almost totally inactive, with only less than 10% of the enzyme activity remaining.

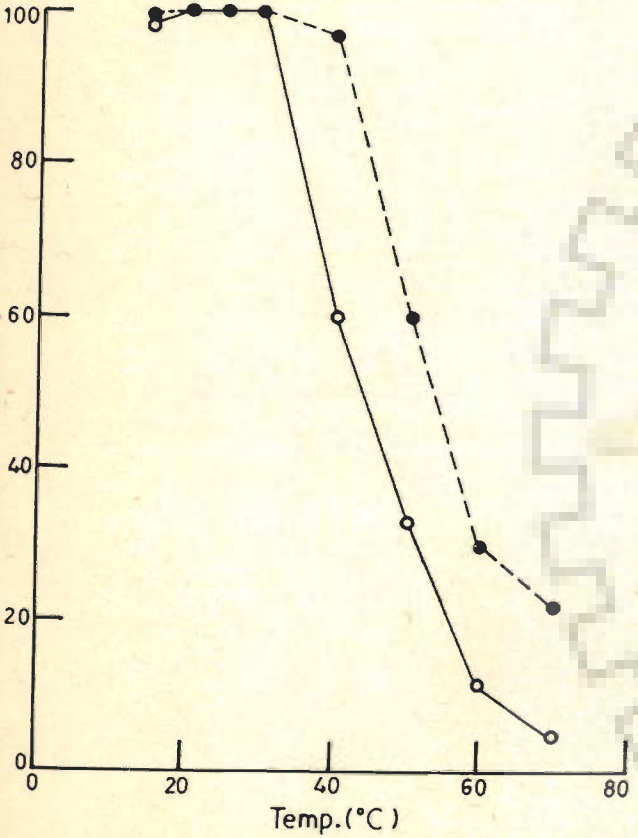
TABLE VII INHIBITION BY $Ag^{+}$ and $Hg^{2+}$

| Metal ions | α-galactosidase I | α-galactosidase IIa | α-galactosidase IIb |
|---|---|---|---|
| $Ag^{+}$ | Competitive $K_i$ = 8 μmol | Competitive $K_i$ = 0.8 μmol | Competitive $K_i$ = 0.5 μmol |
| $Hg^{2+}$ | Competitive $K_i$ = 1.0 μmol | Competitive $K_i$ = 0.6 μmol | Competitive $K_i$ = 0.75 μmol |

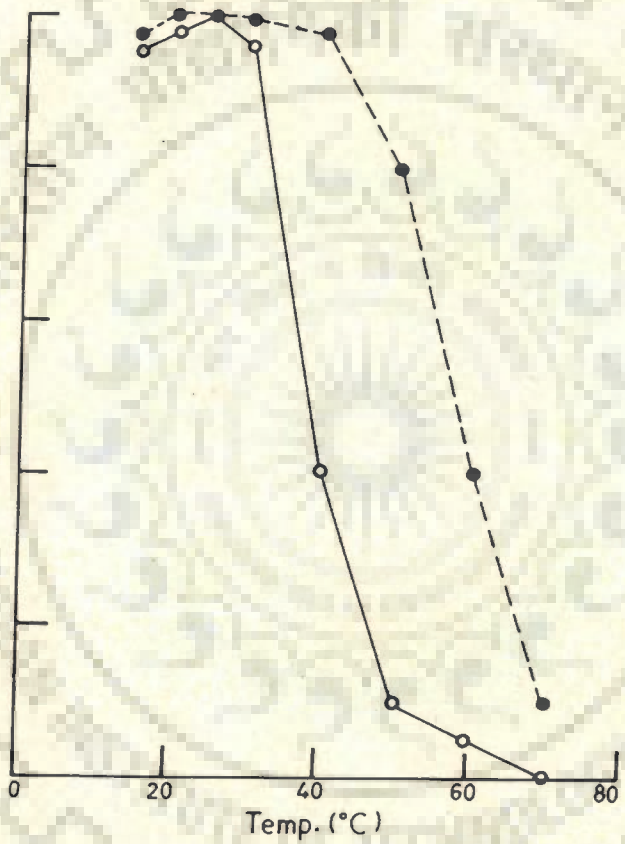
FIG. 12. EFFECT OF TEMPERATURE ON FREE AND IMMOBILIZED CHICKPEA α-GALACTOSIDASE ISOENZYMES

The enzymes were incubated to indicated temperatures at optimum pH for 30 min. Enzyme activity was assayed by standard procedure described in 'Experimental Procecure'. The controls (without heat treatment) were run simultaneously and used as 100% . The percent residual activity was plotted versus temperature. o———o , free enzyme ; •--------• , immobilized enzyme.

(A) ∝-Galactosidase I




(B) ∝-Galactosidase IIa




(C) ∝-Galactosidase II b

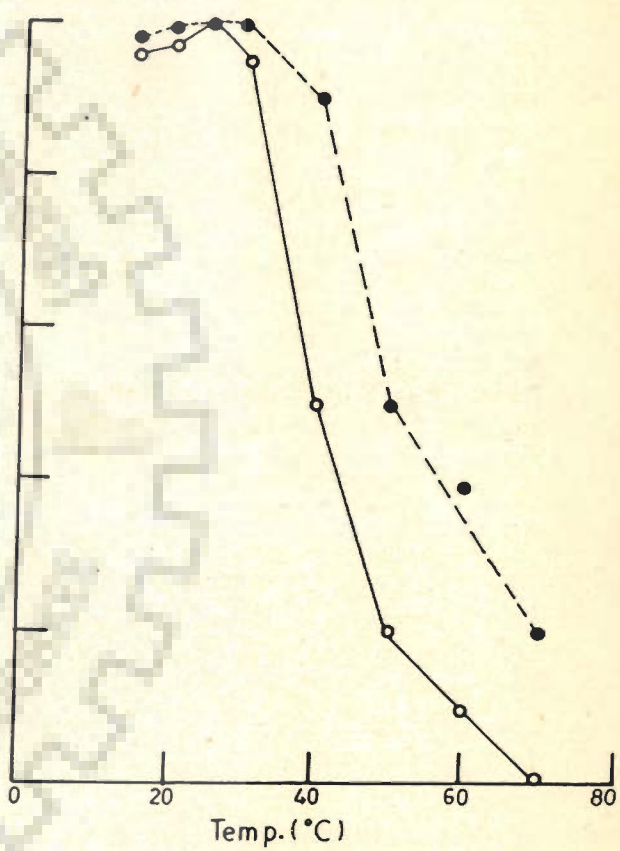
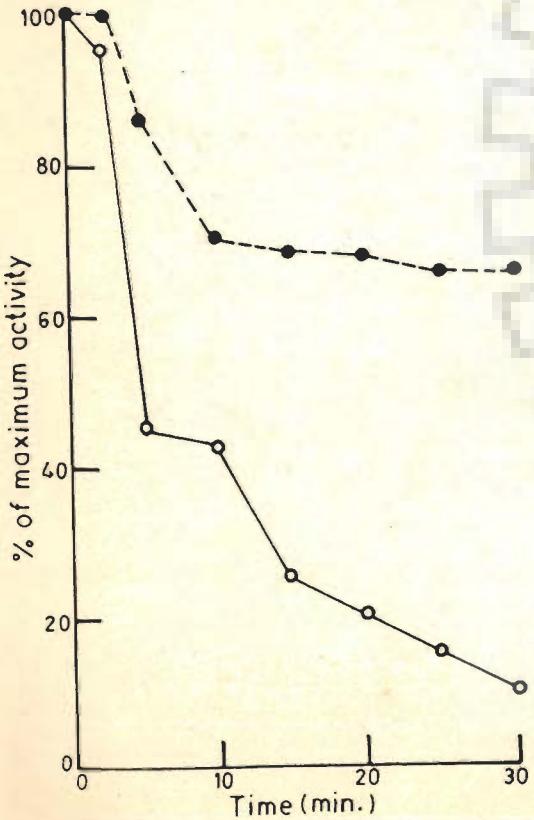


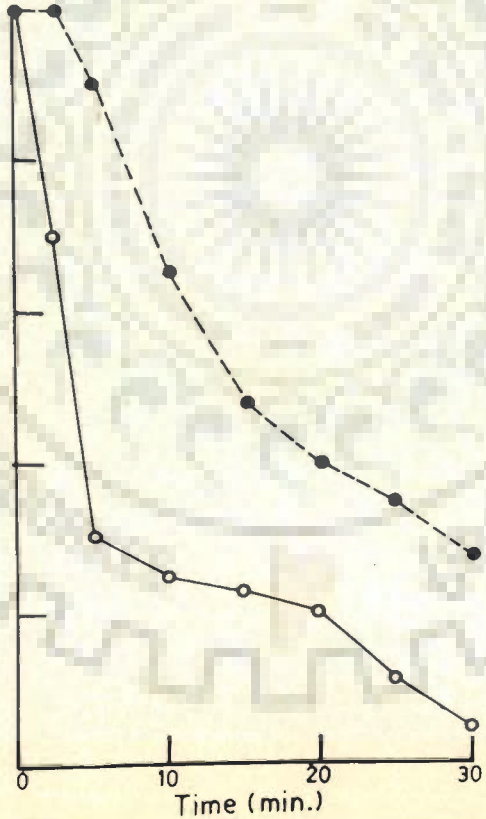
FIG. 13. STABILITY OF FREE AND IMMOBILIZED CHICKPEA α-GALACTOSIDASE ISOENZYMES AT 50°C.

The enzymes were first exposed to 50°C at optimum pH for different time periods (2-30 min) and then cooled in ice bath for 30 minutes. Enzyme activity was assayed by standard procedure described in 'Experimental Procedure'. The controls (without heat treatment) were run simultaneously and used as 100 per cent. The residual per cent activity was plotted versus time (minutes). o——o, free enzyme; •------•, immobilized enzyme.

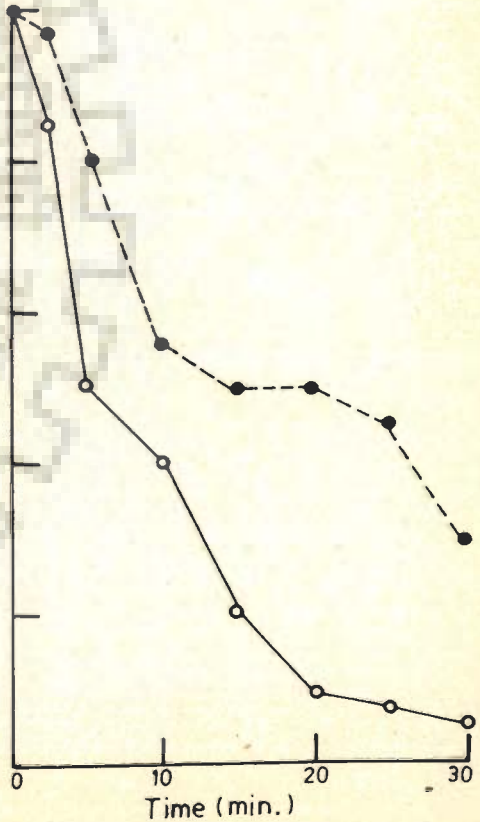
(A) ∝-Galactosidase I




(B) ∝-Galactosidase IIa




(C) ∝-Galactosidase IIb



### 4.5.8 KINETIC PARAMETERS

The values for apparent $K_m$ and $V_{max}$ of various isoenzymes with p-nitrophenyl-α-D galactopyranoside substrate were determined by Lineweaver Burk plots (Fig.14,15,16). $K_m$ values of α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb were found to be 0.36 mM, 0.14 and 0.28 mM with $V_{max}$ values of 5.3 μmol/min/mg, 2.0 μmol/min/mg and 1.0 μmol/min/mg, respectively.

### 4.5.9 INTERACTION CONSTANT

Fig 17 shows the double logarithmic plots of p-nitrophenyl α-D-galactopyranoside concentrations versus $[\frac{V}{V_{max}} - V]$ (Hill's plot). In each experiment, these plots show a linear relationship with a constant slope of one indicating that one molecule of substrate with one molecule of enzyme to form an active enzyme substrate complex and that the phenomenon of coperativity is not present (146).

### 4.5.10 THERMODYNAMIC PARAMETERS

The effect of temperature on the activity of α-galactosidase enzymes was further investigated by determining various thermodynamic parameters. In order to avoid the thermal denaturation the temperature range between 15°C to 40°C was selected. The results are summarized in Table VIII.

FIG. 14. LINEWEAVER-BURK PLOTS OF FREE AND IMMOBILIZED α-GALACTOSIDASE I

Enzyme assay with varying amounts of substrate, p-nitrophenyl α-D-galactopyranoside (100 μmol to 700 μmol) and fixed amount of enzyme protein was carried out as described under 'Experimental Procedure'. $K_m$ and $V_{max}$ values were computed from the intercept on X-axis and Y-axis, respectively. o———o, free enzyme; •———•, immobilized enzyme.

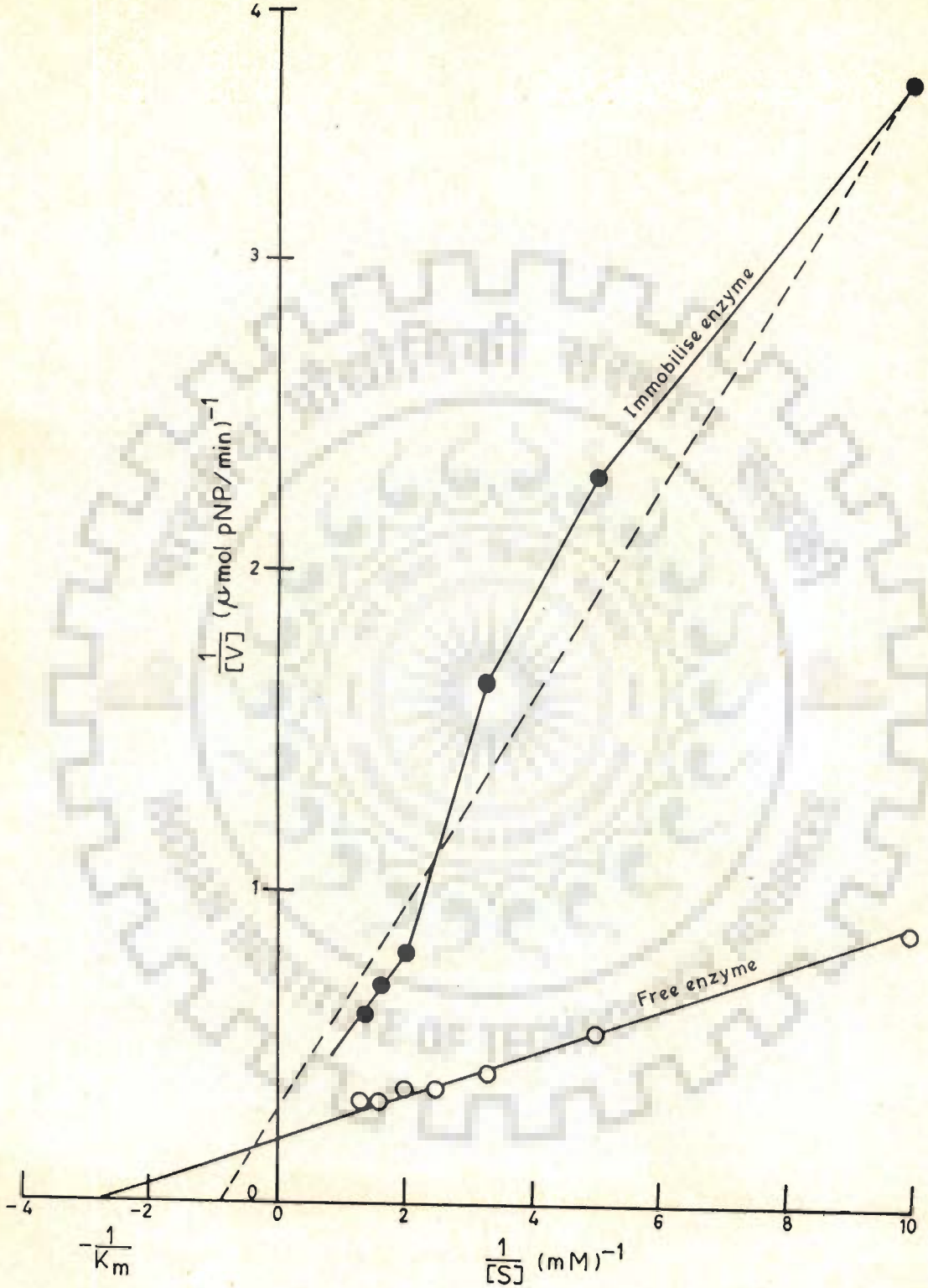

4
3
2
1
0
-4
-2
0
2
4
6
8
10
$\frac{1}{[V]}$ ($\mu$mol pNP/min)$^{-1}$
$-\frac{1}{K_m}$
$\frac{1}{[S]}$ (mM)$^{-1}$
Immobilise enzyme
Free enzyme

FIG.15. LINEWEAVER-BURK PLOTS OF FREE AND IMMOBILIZED α-GALACTOSIDASE IIa.

Enzyme assay with varying amounts of substrate, p-nitrophenyl α-D-galactopyranoside (100 μmol to 700 μmol) and fixed amount of enzyme protein was carried out as described under 'Experimental Procedure'. $K_m$ and $V_{max}$ values were computed from the intercept on X-axis and Y-axis, respectively. o——————o, free enzyme; ●——————●, immobilized enzyme.

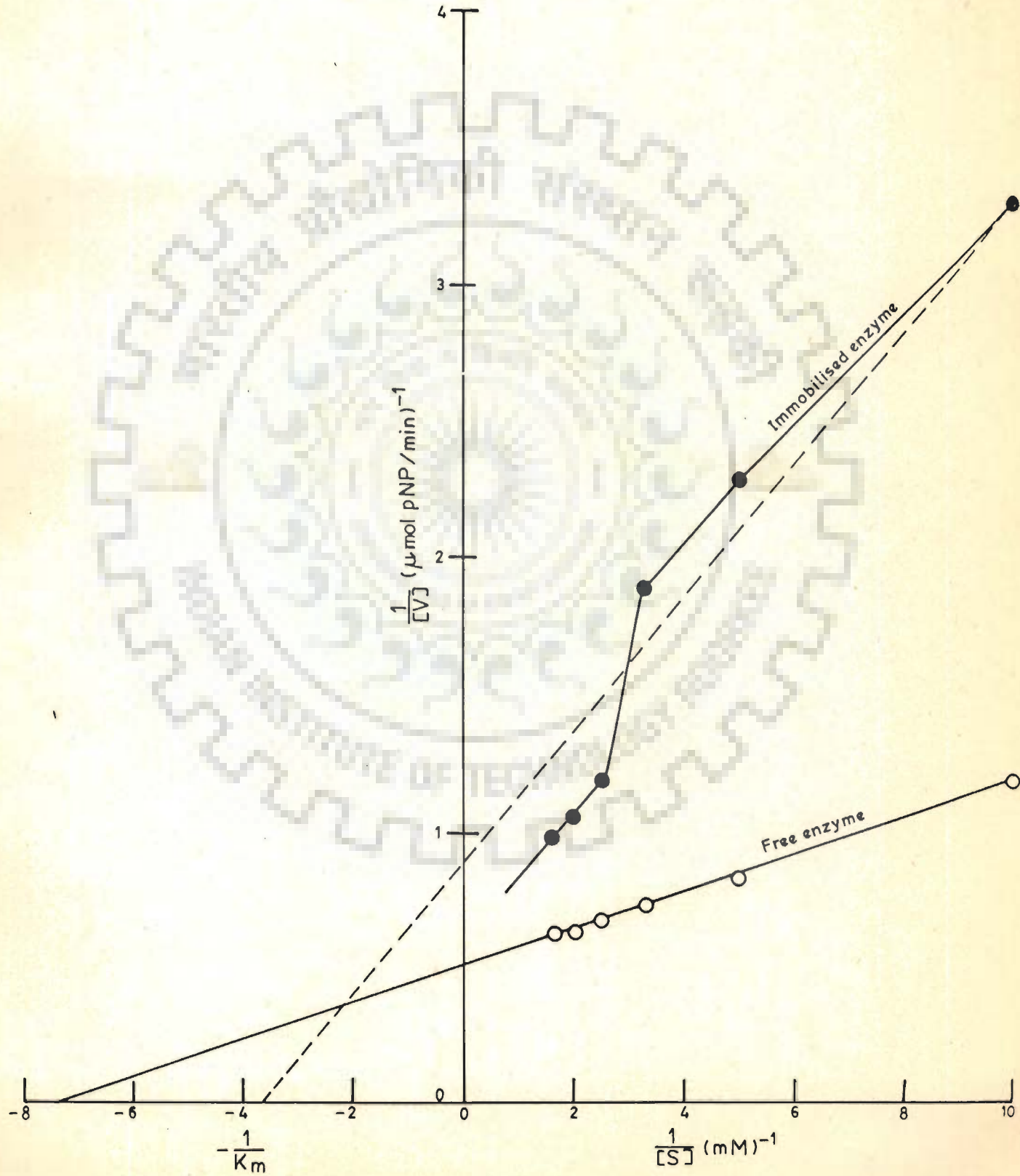

4
3
2
1
0
$\frac{1}{[V]}$ (μmol pNP/min)$^{-1}$
Immobilised enzyme
Free enzyme
-8
-6
-4
-2
0
2
4
6
8
10
$-\frac{1}{Km}$
$\frac{1}{[S]}$ (mM)$^{-1}$

FIG.16. LINEWEAVER-BURK PLOTS OF FREE AND IMMOBILIZED α-GALACTOSIDASE IIb.

Enzyme assay with varying amounts of substrate, p-nitrophenyl α-D-galactophyranoside (100 μmol to 700 μmol) and fixed amount of enzyme protein was carried out as described under 'Experimental Procedure'. $K_m$ and $V_{max}$ values were computed from the intercept on X-axis and Y-axis, respectively. o——————o, free enzyme; ●——————●, immobilized enzyme.

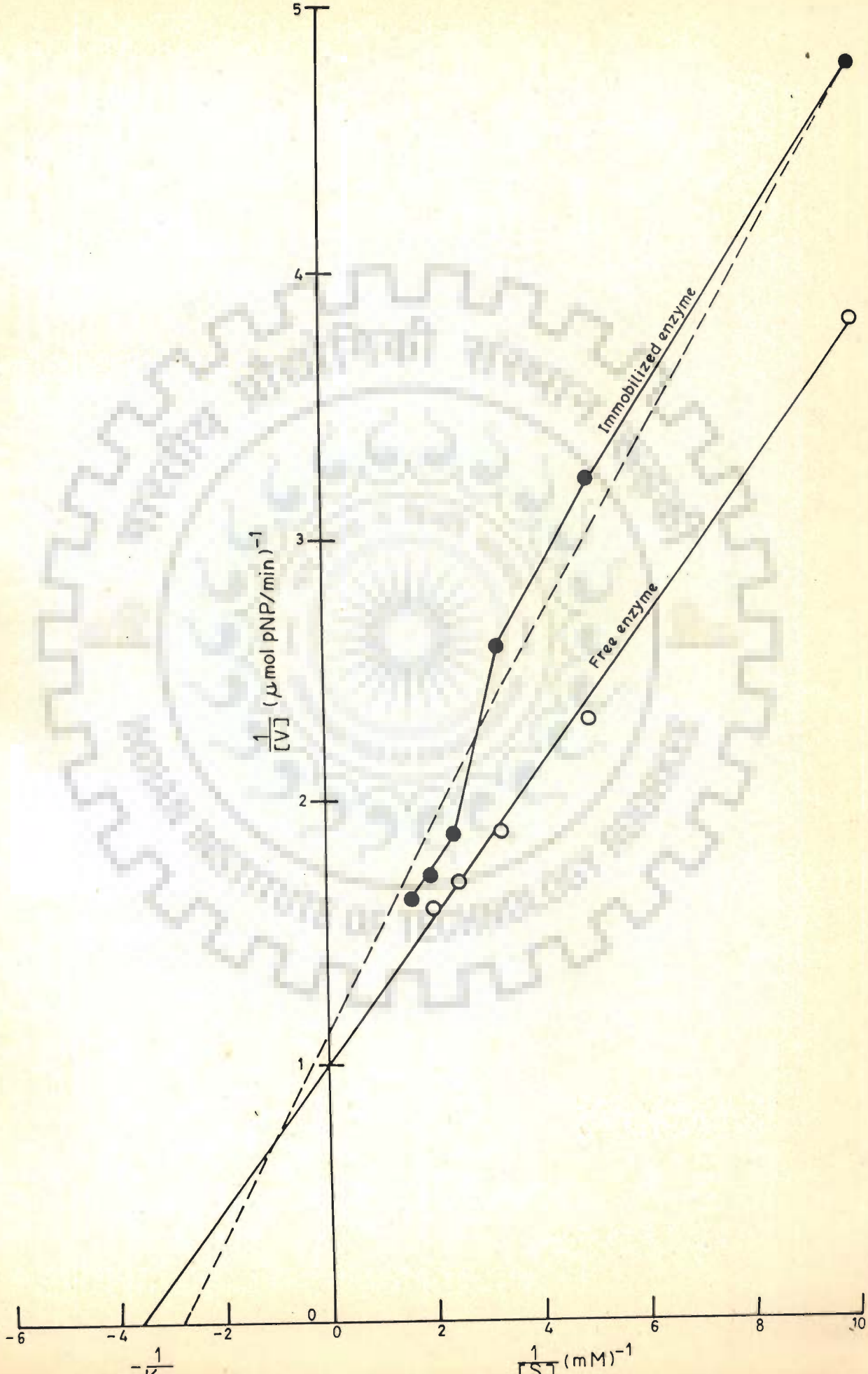

5
4
3
2
1
0
-6
-4
-2
0
2
4
6
8
10
Immobilized enzyme
Free enzyme
$\frac{1}{[V]}$ ($\mu$mol pNP/min)$^{-1}$
$\frac{1}{[S]}$ (mM)$^{-1}$
$-\frac{1}{K}$

FIG.17. HILL'S PLOTS FOR INTERACTION OF α-GALACTOSIDASE IIa AND α-GALACTOSIDASE IIb WITH p-NITDRPHENYL α-D-GALACTOPYRANOSIDE.

Curves were obtained by plotting $\log_{10}\left(\frac{V}{V_{max}-V}\right)$ against log (S) and the number of interacting substrate molecule per enzyme molecule 'n' was determined from the slope of curve.

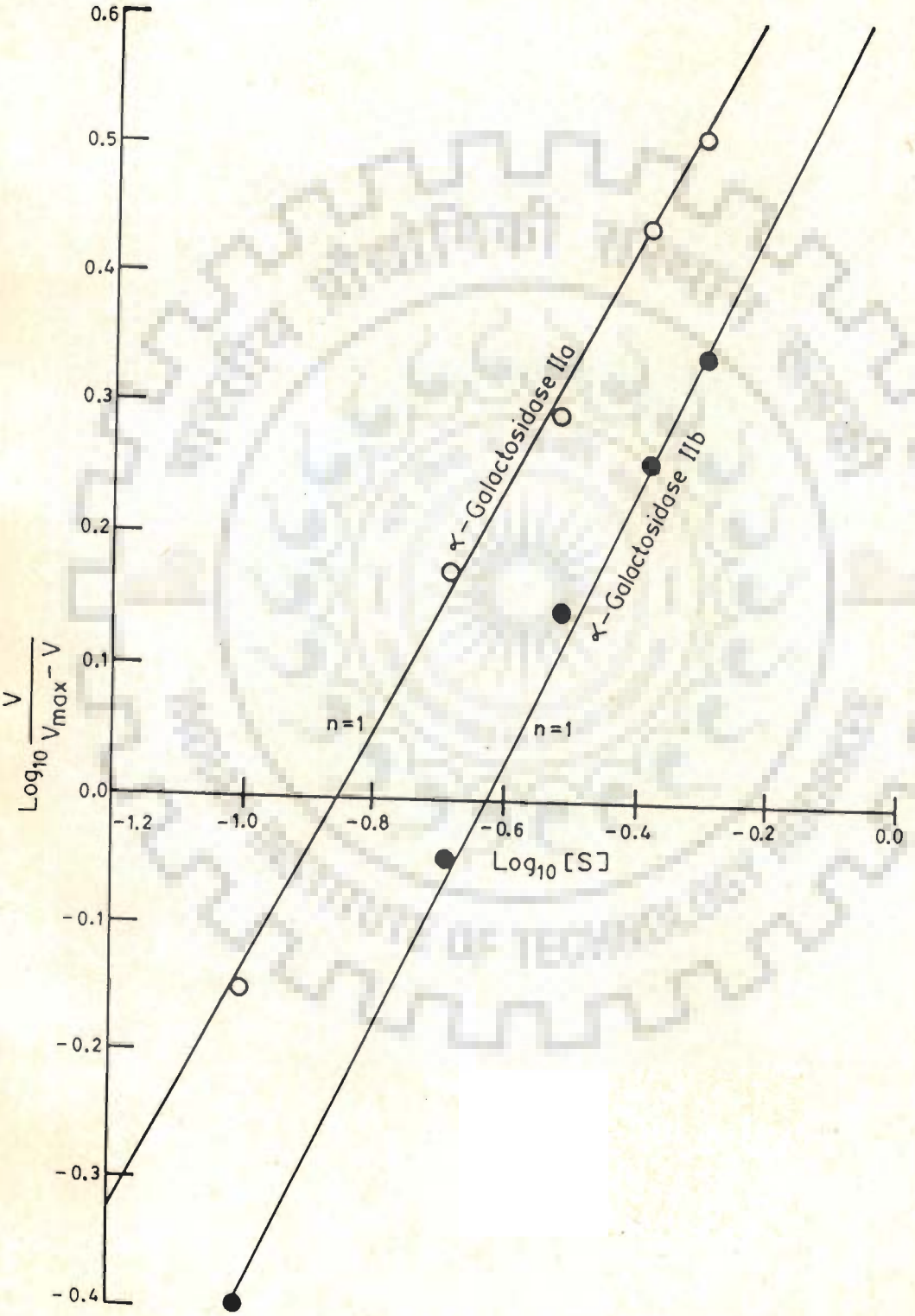

0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
$\log_{10} \frac{V}{V_{max} - V}$
$\log_{10}[S]$
α-Galactosidase IIa
α-Galactosidase IIb
n=1
n=1

TABLE VIII THERMODYNAMIC PARAMETERS FOR THE INTERACTION OF α-GALACTOSIDASE IIa AND IIb WITH p-NITRO PHENYL α-D-GALACTOPYRANOSIDE

| Thermodynamic parameters | α-galactosidase IIa | α-galactosidase IIb |
|---|---|---|
| $Q_{10}$ | 0.83 | 0.72 |
| $E_a$ (Kcal/mol) | 14.64 | 16.64 |
| $\Delta H$ (Kcal/mol) | 15.61 | 11.37 |
| $\Delta G$ at 25°C (Kcal/mol) | 1.17 | 0.77 |
| $\Delta S$ at 25°C (Kcal/mol/Kelvin) | 0.048 | 0.035 |

The value of temperature quotient ($Q_{10}$) for α-galactosidase IIa and α-galactosidase IIb were found to be 0.83 and 0.72, respectively.

Fig.18 shows the Arrhenius plots of $\log_{10} V_{max}$ versus $\frac{1}{T}$ for α-galactosidase IIa and IIb used to determine the energy of activation ($E_a$). The values of $E_a$ computed from the slope of these plots, were found to be 14.64 and 16.64 Kcal/mol, respectively.

Assuming that the $K_m$ values at different temperatures represent the association constant for the formation of enzyme-substrate complex, the thermodynamic parameters $\Delta H$ of α-galactosidase IIa and α-galactosidase IIb as computed from the linear plots of $\frac{1}{T}$ versus $\log_{10} K_m$ (Fig.19), was found to be 15.61 Kcal/mol and 11.37 Kcal/mol, respectively. The positive $\Delta H$ indicates that the hydrolysis of p-nitrophenyl-α-D-galactopyranoside by α-galactosidase IIa and α-galactosidase IIb was endothermic in nature.

The values of free energy change $\Delta G$ and enthalpy change ($\Delta S$) for the interaction between p-nitrophenyl α-D-galactopyranoside and α-galactosidase isoenzymes as a function of temperature are shown in Table IX. These results show that there is a regular decrease in the free energy change with temperature for both α-galactosidase IIa and α-galactosidase IIb. The positive

FIG.18. ARRHENIUS PLOTS OF α-GALACTOSIDASE IIA AND α-GALACTOSIDASE IIb.

Value of 'activation energy' of α-galactosidase IIa and α-galactosidase IIb was calculated from the slope of curves of $\log_{10} V_{max}$ versus 1/T, (T= $^{o}K$). The $V_{max}$ values (μmol p-nitrophenol/min) for each enzyme were obtained by the double reciprocal plots of (1/V) versus (1/S) under optimum assay conditions.

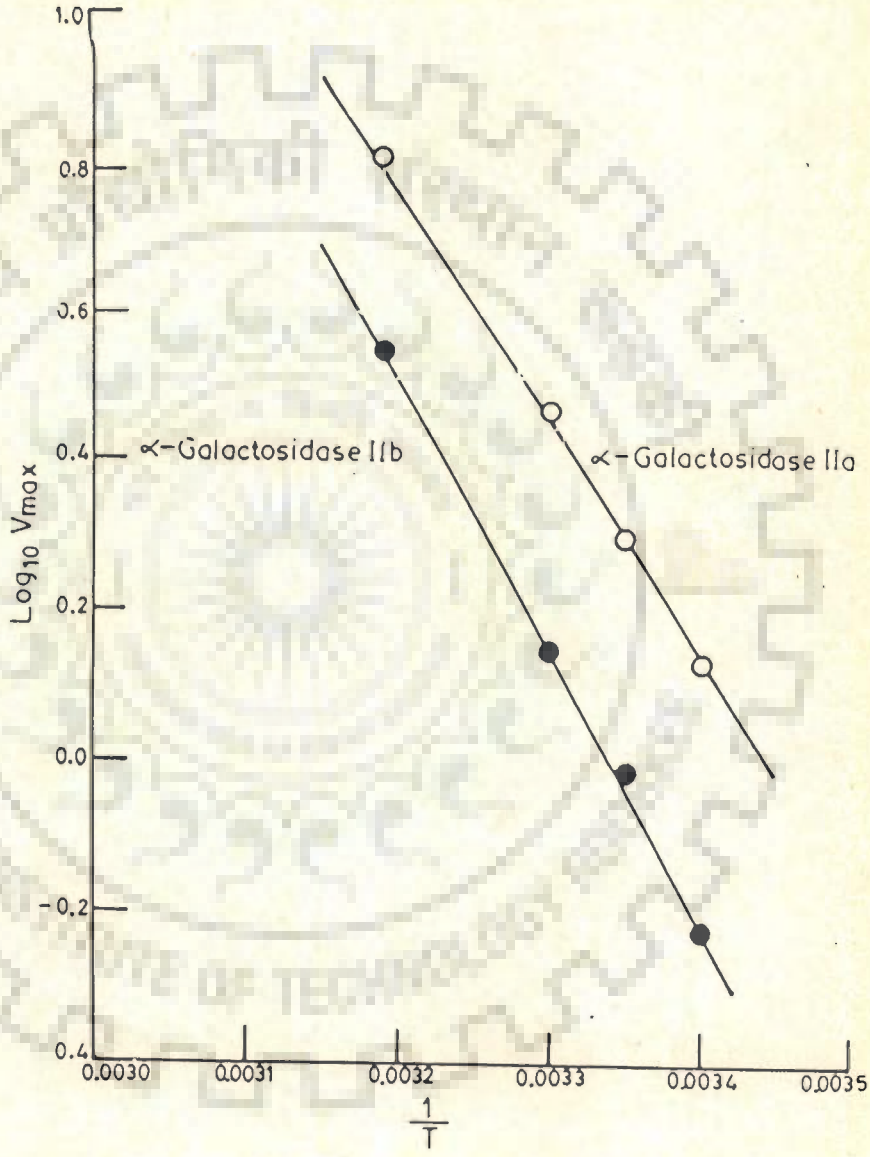

1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
Log10 Vmax
∝-Galactosidase IIb
∝-Galactosidase IIa
0.0030
0.0031
0.0032
0.0033
0.0034
0.0035
1/T

FIG.19. VAN'T HOFF PLOTS OF α-GALACTOSIDASE IIa AND α-GALACTOSIDASE IIb.

Value of 'Free energy change, $\Delta G'$ of α-galactosidase IIa and α-galactosidase IIb was calculated from the slope of curves of $\log_{10} K_m$ versus $1/T (T = {}^{o}K)$. (Slope of the curve is equal to $-\Delta H/2.3R$). The $K_m$ values for each enzyme were obtained by the double reciprocal plots of (1/V) versus (1/S) under optimum assay conditions.

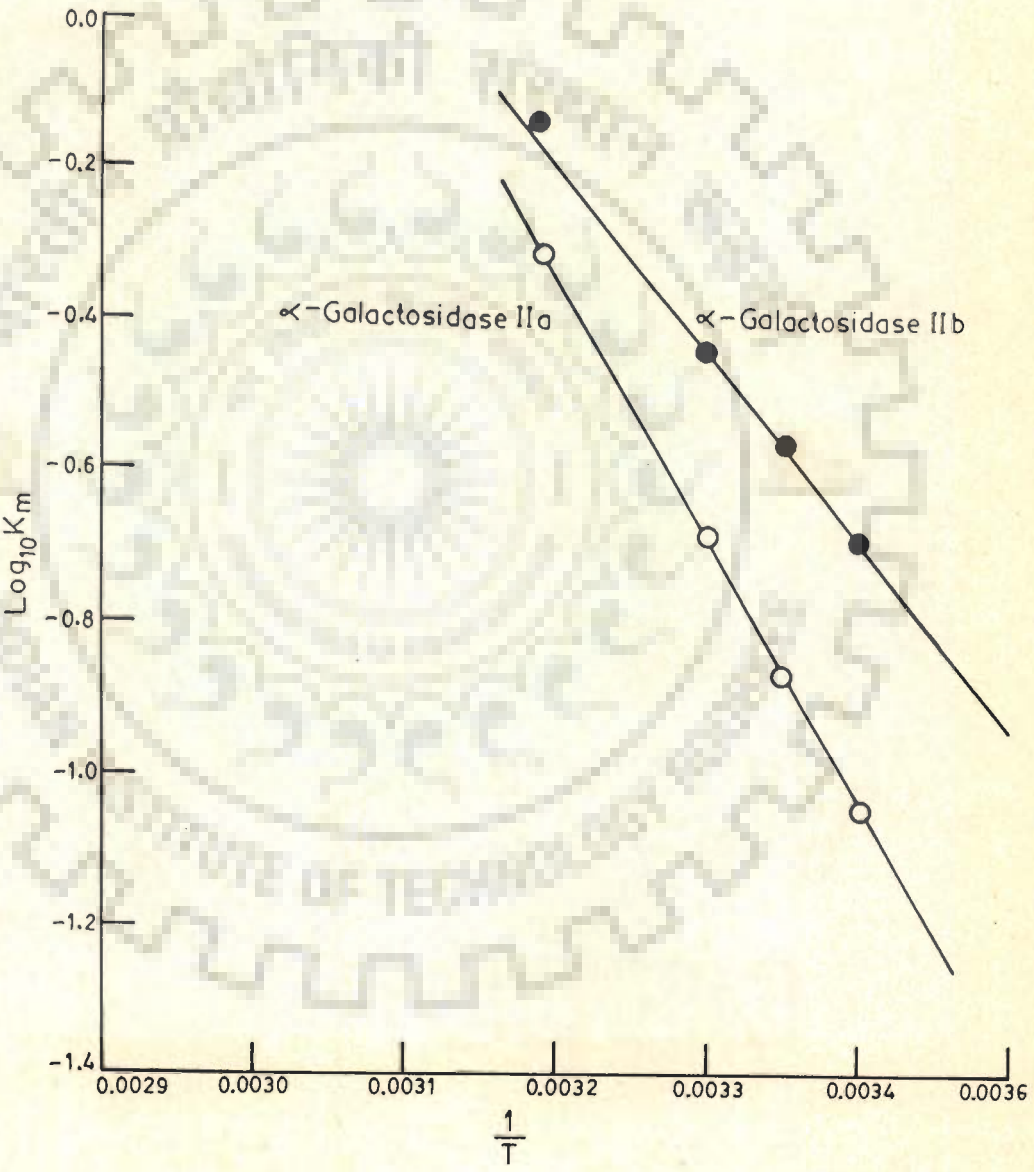

0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
$Log_{10} K_m$
∝-Galactosidase IIa
∝-Galactosidase IIb
0.0029
0.0030
0.0031
0.0032
0.0033
0.0034
0.0036
$\frac{1}{T}$

TABLE IX FREE ENERGY CHANGE ($\Delta G$) AND ENTROPY CHANGE ($\Delta S$) FOR THE INTERACTION BETWEEN p-NITROPHENYL α-D-GALACTOPYRANOSIDE AND α-GALACTOSIDASE IIa AND IIb AT DIFFERENT TEMPERATURES

Enthalpy change ($\Delta H$) was calculated by Van't Hoff plot; free energy change ($\Delta G$) and Entropy change ($\Delta S$) were calculated by following equations:

$$\Delta G = -2.303\ RT \log_{10} {}^{0}K_m \text{ and}$$

$$\Delta S = \frac{\Delta H - \Delta G}{\Delta T}, \text{ respectively}$$

| Temperature | ΔG(Kcal/mol) | | ΔS(Kcal/mol/Kelvin) | |
|---|---|---|---|---|
| | α-galactosidase | | α-galactosidase | |
| ($^{o}K$) | IIa | IIb | IIa | IIb |
| 293 | 1.39 | 1.05 | 0.0485 | 0.0352 |
| 298 | 1.17 | 0.77 | 0.0484 | 0.0355 |
| 303 | 0.95 | 0.61 | 0.0483 | 0.0355 |
| 313 | 0.44 | 0.24 | 0.0484 | 0.0355 |

$\Delta G$ value once again indicates that the reaction is endogenic.

There is no significant change in $\Delta S$ values of α-galactosidase Isoenzymes (α-galactosidase IIa and α-galactosidase IIb) with change in temperature. The constant $\Delta S$ values indicate that no significant conformational change occurs in the enzyme active site as a result of heat treatment between temperature range indicated. However, the $\Delta S$ values for α-galactosidase IIa and α-galactosidase IIb were found to be different, indicating that conformational changes which the two isoenzymes undergo during the enzyme substrate complex formation are different.

4.5.11 STORAGE STABILITY

The purified α-galactosidase isoenzymes were found to lose the enzymatic activity if stored at optimum pH at 4°C. After 10 days of storage α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb were having 49% , 9% , and 14% of the maximum activities, respectively (Table X). Results given in Table XI show that when α-galactosidase II (low molecular weight form) obtained after Sephadex G-150 gel filtration containing a mixture of α-galactosidase IIa and IIb was stored under these conditions the specific activity increased considerably.

TABLE-X. A COMPARISION OF STABILITY OF FREE AND IMMOBILIZED ISOENZYMES OF α-GALACTOSIDASE

The immobilized enzyme and the free enzyme preparations were stored for varying time periods at 4°C and then assayed in the routine way as described in 'Experimental Procedures'

| Period of storage (days) | Per cent residual activity | | | | | |
|---|---|---|---|---|---|---|
| | α-galactosidase I | | α-galactosidase IIa | | α-galactosidase IIb | |
| | Free | Immobilized | Free | Immobilized | Free | Immobilized |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5 | 75 | 80 | 40 | 80 | 55 | 95 |
| 10 | 49 | 70 | 9 | 50 | 14 | 60 |
| 15 | 12 | 50 | 2 | 30 | 10 | 40 |
| 20 | 0 | 15 | 0 | 8 | 2 | 10 |

TABLE XI INTERCONVERSION OF LOW MOLECULAR FORM INTO HIGH MOLECULAR FORM α-GALACTOSIDASE

The α-galactosidase II obtained after Sephadex G-150 gel filtration was stored for varying time periods at 4°C 0.05 H in acetate buffer, pH 5.2, and then assayed in the routine way as described in Experimental Procedure

| Period of storage (days) | Amount of protein (mg) | Total activity (units) | Specific activity (units/mg of protein |
|---|---|---|---|
| 0 | 3.25 | 136 | 41.8 |
| 10 | 3.25 | 150 | 46.1 |
| 25 | 3.25 | 176 | 54.1 |
| 40 | 3.25 | 226 | 69.5 |
| 55 | 3.25 | 308 | 94.7 |
| 70 | 3.25 | 366 | 112.6 |

## 4.5.12 GLYCOPROTEIN NATURE OF α-GALACTOSIDASES

The purified α-galactosidase IIa and IIb were found to be glycoproteins having 9.4 and 29.5% (w/w) carbohydrate moiety, respectively.

The purified glycoprotein enzyme samples when treated with endo-N-acetyl-β-D-glucosaminidase, which cleaves specifically the glycosidic bond between two adjacent N-acetylglucosamine residues, released nearly 75% of the carbohydrate present in the glycoprotein (Table XII). These results suggested that the oligosaccharide was attached to the polypeptide backbone through N-glycosidic linkage between the amide nitrogen of asparagine residue and the $C_1$ of N-acetylglucosamine , and that the chitoboise unit was present in the oligosaccharide. The analysis of monosaccharide composition of the oligosaccharide moiety of α-galactosidase IIa and α-galactosidase IIb by HPLC showed the presence of N-acetylglucosamine and mannose (Table XIII). A rough estimate gave an approximate molar ratio of N-acetyl glucosamine to mannose of 2:9 to 2:15. These results indicated that the oligosaccharide moiety of the α-galactosidase IIa and IIb glycoprotein enzymes was of the high mannose type containing 9 to 15 mannose units. It may be mentioned,

TABLE XII - CLEAVAGE OF CARBOHYDRATE MOIETY FROM α-GALACTOSIDASE IIa AND IIb BY TREATMENT WITH ENDO-N-ACETYL-β-D-GLUCOSAMINIDASE

The glycoprotein enzymes were treated with endo-N-acetyl-β-D-glucosaminidase as described in 'Experimental Procedures'. The reaction was stopped by heating in boiling water bath for 5 min followed by the addition of 10% trichloroacetic acid. After cooling the reaction mixture was centrifuged. The pellet and the supernatant fractions were assayed for carbohydrate content. Control were without endo-N-acetylglucosaminidase and were processed in identical way.

| Duration of enzyme treatment (h) | Per cent carbohydrate released | | | |
|---|---|---|---|---|
| | α-galactosidase IIa | | α-galactosidase IIb | |
| | Pellet | Supernatant | Pellet | Supernatant |
| 0 | 95-100 | nil | 95-100 | nil |
| 6 | 60±4 | 34±2 | 60±4 | 40±2 |
| 12 | 36±3 | 59±5 | 33±3 | 64±5 |
| 18 | 28±4 | 68±5 | 26±4 | 70±5 |
| 24 | 25±5 | 72±6 | 26±3 | 75±6 |

TABLE XIII - ANALYSIS OF MONOSACCHARIDES BY HPLC

The glycoprotein samples were hydrolyzed and prepared for HPLC analysis as described in 'Experimental Procedures'. The samples (20 µl) were injected on Shimadzu LC-4A system on Zorbax C-18 column. The fractionation was performed with the mobile phase of acetonitrile -water (90:10, v/v) at a flow rate of 1.5 ml/min. The monosaccharides were detected at 195nm wavelength and K values were calculated with respect to the injection peak time (K' value = Retention time/injection peak).

| Reference compound | Standard sample | | α-galactosidase IIa | | α-galactosidase IIb | |
|---|---|---|---|---|---|---|
| | Retention time (min) | K' value | Retention time (min) | K' value | Retention time (min) | K' value |
| N-Acetylglucosamine | 10.8 | 5.7 | 10.84 | 5.65 | 10.82 | 5.64 |
| Mannose | 5.7 | 3.0 | 5.75 | 3.01 | 5.74 | 3.01 |
| Glucose | 4.4 | 2.32 | * | * | * | * |

* Only a very minute peak was detected.

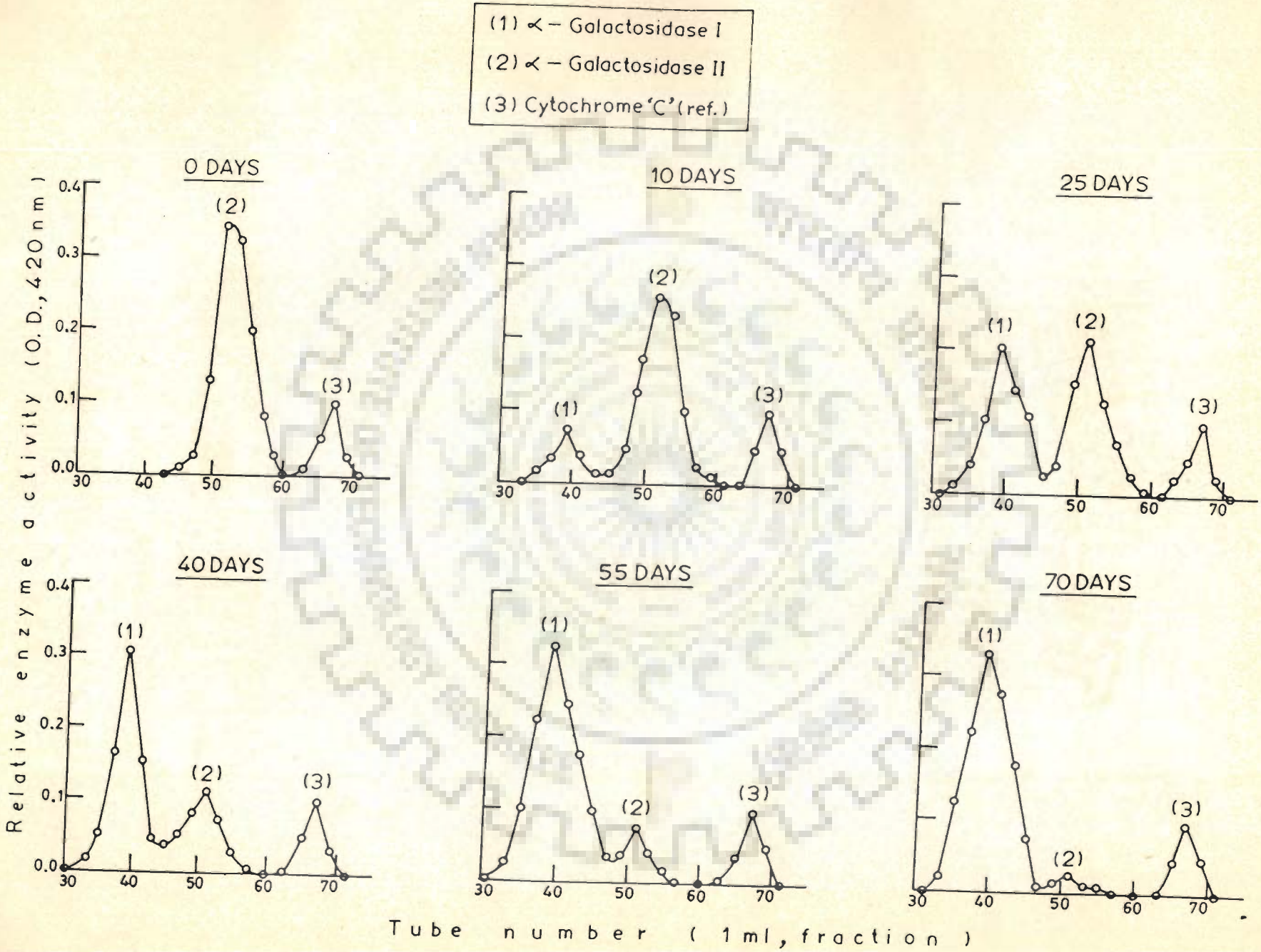
however, that these are highly qualitative estimates and more accurate estimations would be necessary for the exact size of the oligosaccharide moiety.

## 4.6 INTER-RELATIONSHIP OF HIGH AND LOW MOLECULAR WEIGHT FORMS OF α-GALACTOSIDASE

The results given in Table XI clearly show that the partially purified α-galactosidase II obtained after Sephadex G-150 gel filtration when stored at 4°C in 0.05 M acetate buffer, pH 5.2, over a period of about two and a half months, the specific activity of the enzyme increased several folds. Further, when the Sephadex gel filtration of the high specific activity preparation was performed under the identical conditions an additional peak corresponding to high molecular form of the enzyme (Mr=149,000 daltons) was obtained. These results show the emergence of high molecular enzyme peak during the storage period. As can be seen from the results in Fig 20, the activity level of α-galactosidase I increases with increasing storage period accompanied with a concomitant decline in the level of α-galactosidase II activity. After 70 days of storage, it was found that almost entire enzyme II was converted into α-galactosidase I. Interestingly enough, when α-galactosidase IIa and α-galactosidase IIb were stored separately there was no interconversion of low molecular form α-galactosidase to the high molecular form of the enzyme.

FIG. 20. CONVERSION OF LOW MOLECULAR WEIGHT α-GALACTOSIDASE TO A HIGH MOLECULAR WEIGHT FORM IN VITRO.

The α-galactosidase II obtained after Sephadex gel filtration (Peak II) was stored at 4°C in 0.05 M sodium acetate buffer pH 5.2, for the indicated period of time and the stored enzyme was analyzed by gel filtration on Sephadex G-150.


(1) ∝ – Galactosidase I
(2) ∝ – Galactosidase II
(3) Cytochrome 'C' (ref.)
0 DAYS
10 DAYS
25 DAYS
40 DAYS
55 DAYS
70 DAYS
Relative enzyme activity (O.D., 420 nm)
Tube number ( 1 ml, fraction )
0.0
0.1
0.2
0.3
0.4
30
40
50
60
70
(1)
(2)
(3)

The properties of the newly formed (reconstituted) enzyme were compared with α-galactosidase I. It was found that the properties of both the enzymes were very comparable and were quite distinct from the α-galactosidase IIa and α-galactosidase IIb (Table XIV). The carbohydrate content of the reconstituted high molecular form of α-galactosidase were found to be 25% .

## 4.7 IMMOBILIZATION

The α-galactosidase isoenzymes were immobilized by entrapment in the form of polymerized calcium alginate beads. The calcium alginate beads containing immobilized enzyme are shown in Fig 21. The average diameter of beads was 3 mm. The enzyme entrapped in beads was used as immobilized enzyme for further studies.

### 4.7.1 pH PROFILES OF IMMOBILIZED α-GALACTOSIDASE ISOENZYMES

The pH profiles for the free (non-immobilized) and the immobilized enzyme derivatives were found identical (Fig 11) with no shift in the optimum pH values. However, the immobilized enzymes exhibited higher pH tolerance in both lower (pH 3.5 - 5.0) and higher (pH 5.0-7.5) pH range. For instance, at a pH of 3.5 the free α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb, after 60 minutes treatment retained about 61% , 58% and 25% of their maximum activity,

TABLE XIV - A COMPARISION OF NATURALLY OCCURRING α-GALACTOSIDASE ISOENZYMES AND THE RECONSTITUTED HIGH MOLECULAR FORM FORMED FROM α-GALACTOSIDASE II

| Properties | α-glactosidase I | α-galactosidase IIa | α-galactosidase IIb | High molenular form of α-galactosidase formed from α-galactosidase II |
|---|---|---|---|---|
| Optimum pH | 5.2 | 5-5.2 | 5.5 | 5-5.5 |
| Optimum temp ($^{o}C$) | 25-30 | 25 | 25 | 30 |
| Molecular weight (daltons) | 149,600 | 56,000 | 50,000 | 149,000 |
| $K_m$ (mM) | 0.36 | 0.14 | 0.28 | 0.34 |
| $V_{max}$ (μmol/min/mg) | 5.3 | 2.0 | 1.0 | 5.2 |
| Carbohydrate (% w/w) | n.d. | 9.4 | 29.5 | 25 |

n.d. = not determined.

FIG.21. IMMOBILIZED CALCIUM ALGINATE BEADS.

The α-galactosidase isoenzymes were immobilized by entrapment in the form of polymerized calcium alginate beads. For this 4.0% sodium alginate solution containing 0.4% egg albumin, 0.25% glutaraldehyde with sufficient enzyme was sprayed on to a 0.4 M calcium chloride solution. After 2 h of standing immobilized beads of an average diameter of 3 mm are formed.

80%. activity at pH 7.0 and α-galactosidase IIa an
were more active under these conditions than the
[illegible]s.

[illegible] PROFILES

[illegible] optimum temperature for immobilized
galacto[illegible] and IIb was from 20°C to 30
(Fig [illegible]) found that the immobilized enzy
were more stable at higher temperature than that
free enzyme. For instance, at 60°C free α-gal

while immobilized α-galactosidase I, α-galactosidase IIa and IIb under identical conditions retained 70%, 80% and 60% of their maximum activity. Similarly, at pH 7.0 the free α-galactosidase I become completely inactive while α-galactosidase IIa and α-galactosidase IIb lost about 30% of the enzymatic activity. In contrast, the immobilized α-galactosidase I retained 30% activity at pH 7.0 and α-galactosidase IIa and IIb were more active under these conditions than the free enzymes.

4.7.2 THERMAL PROFILES

The optimum temperature for immobilized α-galactosidase I, IIa and IIb was from 20°C to 30°C (Fig 12). It was found that the immobilized enzymes were more stable at higher temperature than that of free enzyme. For instance, at 60°C free α-galactosidase I, IIa and IIb were having only 10%, 5% and 10% of their maximum activities while immobilized α-galactosidase I, α-galactosidase IIa, IIb showed 30%, 40% and 40% of their maximum activity, respectively.

The immobilized enzyme derivatives were more stable than the soluble enzyme at 50°C (Fig 13). It was found that soluble α-galactosidases show a rapid decline in the enzymatic activity. Only 45%, 30% and 50% of the maximum activities have been retained by soluble

α-galactosidase I, IIa and IIb just after 5 minutes of heat treatment at 50°C while immobilized α-galactosidase I, IIa and IIb retained 85%, 90% and 80% of their maximum activities. On treatment for 30 min at 50°C, the soluble α-galactosidase I, IIa and IIb became almost inactive with only 10%, 5% and 5% of activity while under identical conditions immobilized α-galactosidase I, IIa and IIb still retained 65%, 60% and 30% of maximum activities, respectively.

4.7.3 KINETIC PARAMETERS

The Lineweaver-Burk plots for the immobilized enzymes with p-nitrophenyl-α-D-galactopyranoside substrate are shown in Fig 14, 15, 16. As can be seen the immobilized enzymes do not follow the Michaelis-Menten kinetics as the reciprocals of 1/[S] and 1/[V] give the sigmoid curves instead of straight lines. Therefore, the correct values for $K_m$ and $V_{max}$ for the immobilized enzymes could not be determined. However, using the best fitting linear plots $K_m$ and $V_{max}$ values were measured. A comparison of these results (Table XV) show higher $K_m$ values than that of free enzymes.

4.7.4 STORAGE STABILITY

Although immobilized α-galactosidase isoenzymes were also lose enzymatic activity if stored at optimum pH at 4°C, the results show that immobilized enzyme preparations were more stable than the soluble enzyme

TABLE XV - A COMPARISION OF PROPERTIES OF IMMOBILIZED AND THE FREE ISOENZYMES OF α-GALACTOSIDASE

| Properties | α-galactosidase I | | α-galactosidase IIa | | α-galactosidase IIb | |
|---|---|---|---|---|---|---|
| | Free | Immobilized | Free | Immobilized | Free | Immobilized |
| Optimum pH | 5.2 | 5.2 | 5-5.2 | 5-5.2 | 5.5 | 5.5 |
| Optimum temperature (°C) | 25-30 | 20-30 | 25 | 20-30 | 25 | 20-30 |
| $K_m$ (mM) | 0.36 | 1.0 | 0.13 | 0.28 | 0.27 | 0.36 |
| $V_{max}$ (μmol/min/mg) | 5.26 | 3.3 | 2.0 | 1.1 | 0.99 | 0.9 |

(Table X). For instance, after 10 days of storage soluble α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb were having 49%, 9% and 14% of their maximum activities while immobilized enzymes showed 70%, 50% and 60% of the maximum activity, respectively.

## 4.8 DEVELOPMENTAL CHANGES OF α-GALACTOSIDASE ISOENZYMES IN BLACK VARIETY OF CICER-ARIETINUM SEEDS DURING GERMINATION

The effect of germination period on the total α-galactosidase activity and also on its molecular forms was studied. Results are shown in Fig 2. It was found that α-galactosidase present in dormant seeds tend to increase with germination period upto the 6th day of germination, thereafter the enzyme activity rapidly declined.

In order to investigate the developmental pattern of the different molecular forms of α-galactosidase, the high and low molecular forms of α-galactosidase were first separated by Sephadex G-150 gel filtration and changes in their relative concentrations were determined at various stages showing the mode of development of α-galactosidase I and II in germinating cotyledons of chickpea seeds (Fig 22). Data shown in Fig 23 indicated that during the process of germination the relative

FIG.22. SEPHADEX G-150 GEL FILTRATION PATTERNS OF $\alpha$-GALACTOSIDASE ACTIVITY OF BLACK VARIETY CHICKPEA SEEDS DURING GERMINATION.

0.3 - 0.7 ammonium sulphate fractions in 0.05 M sodium acetate buffer, pH 5.2, obtained from different periods of germination of black variety chickpea seeds were eluted from a Sephadex G-150 column at a flow rate of 0.1 ml/ min. Aliquots (0.1 ml) from every other fractions were tested for enzymatic activity as described in 'Experimental Procedure'.

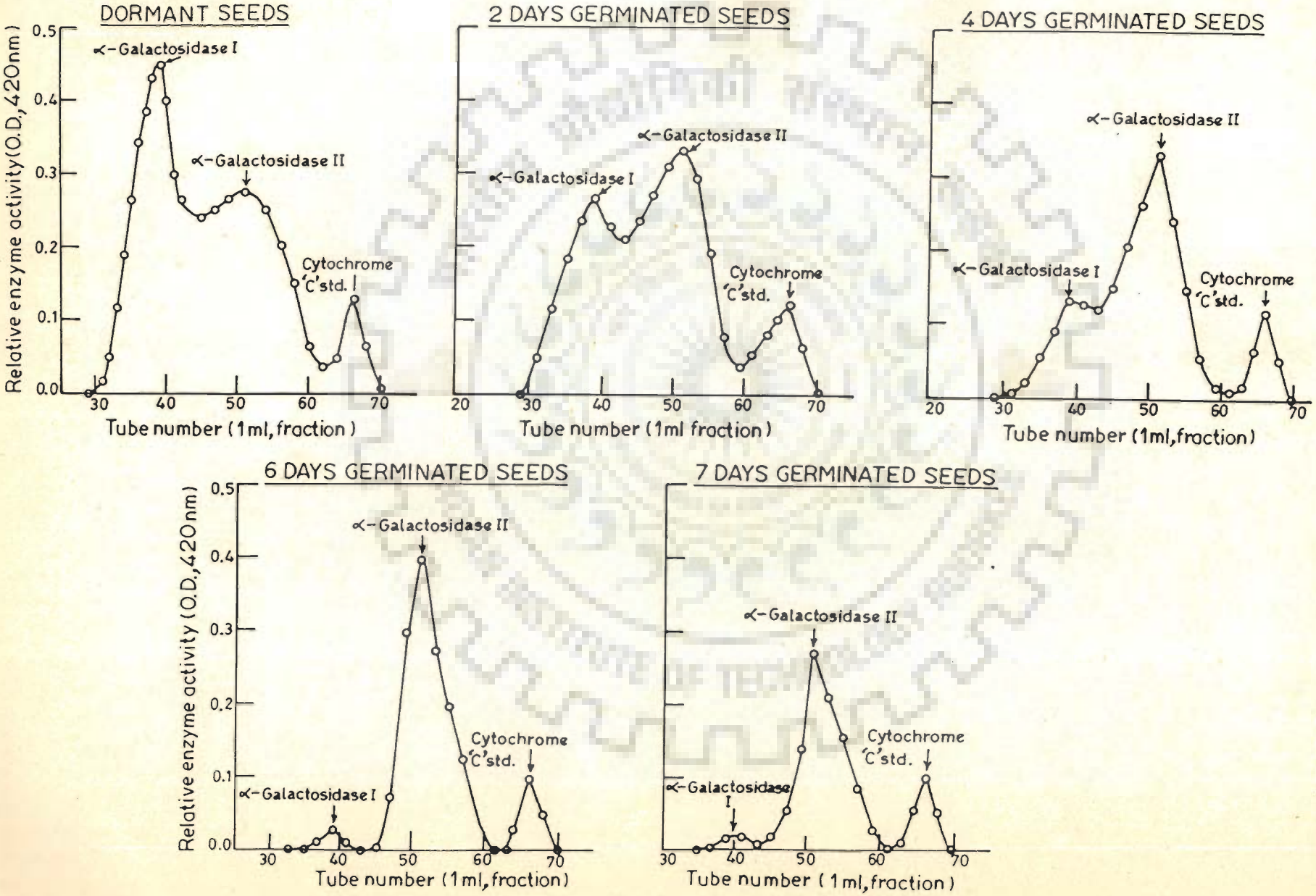
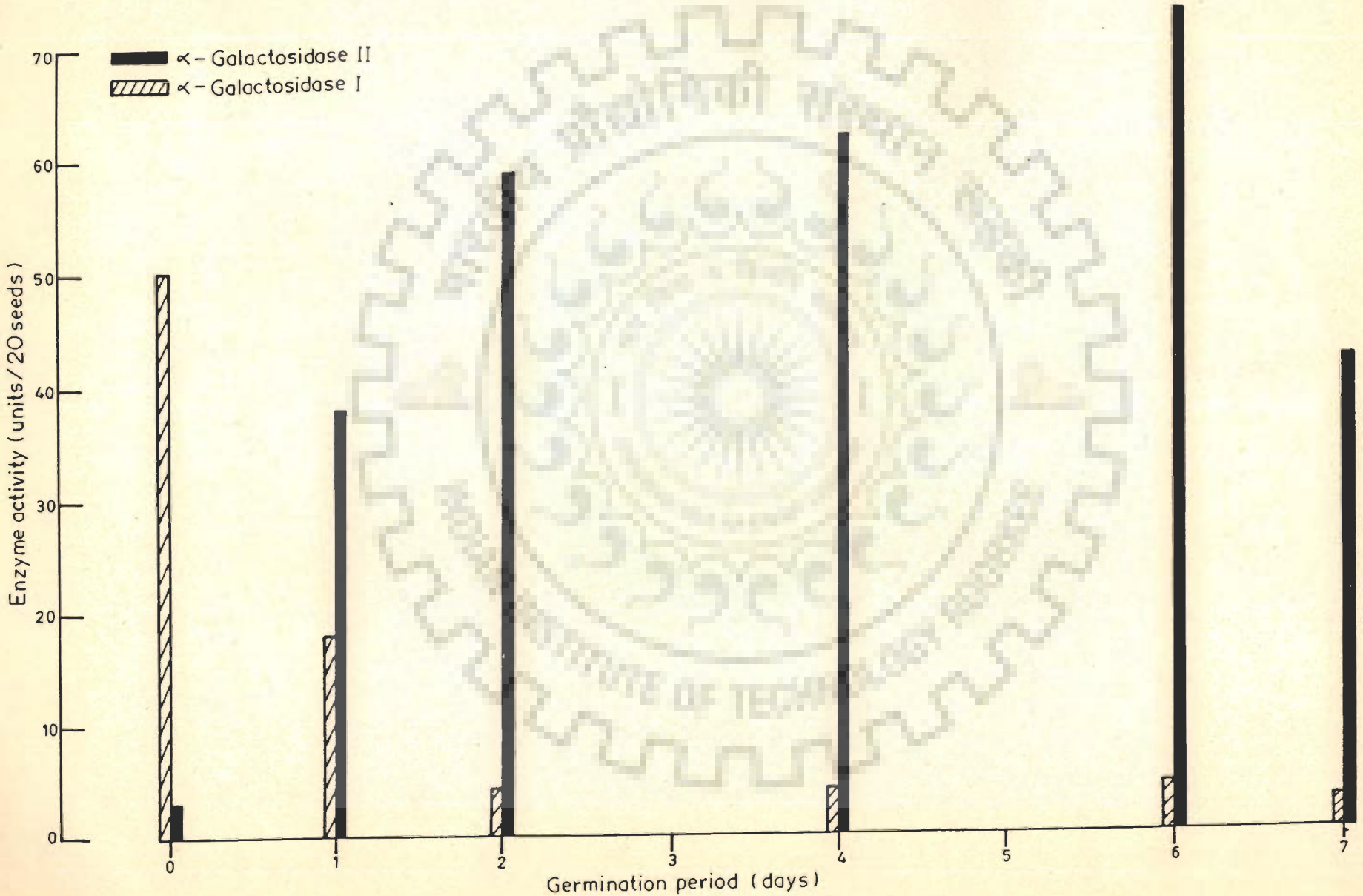

DORMANT SEEDS
Relative enzyme activity(O.D.,420nm)
0.5
0.4
0.3
0.2
0.1
0.0
∝-Galactosidase I
∝-Galactosidase II
Cytochrome 'C'std.
30
40
50
60
70
Tube number (1ml, fraction)
2 DAYS GERMINATED SEEDS
∝-Galactosidase II
∝-Galactosidase I
Cytochrome 'C'std.
20
30
40
50
60
70
Tube number (1ml fraction)
4 DAYS GERMINATED SEEDS
∝-Galactosidase II
∝-Galactosidase I
Cytochrome 'C'std.
20
30
40
50
60
70
Tube number (1ml, fraction)
6 DAYS GERMINATED SEEDS
Relative enzyme activity (O.D.,420nm)
0.5
0.4
0.3
0.2
0.1
0.0
∝-Galactosidase II
Cytochrome 'C'std.
∝-Galactosidase I
30
40
50
60
70
Tube number (1ml, fraction)
7 DAYS GERMINATED SEEDS
∝-Galactosidase II
Cytochrome 'C'std.
∝-Galactosidase I
30
40
50
60
70
Tube number (1ml, fraction)

FIG.23. RELATIVE CHANGES IN α-GALACTOSIDASE I AND α-GALACTOSIDASE II ACTIVITIES DURING GERMINATION OF BLACK VARIETY CHICKPEA SEEDS.

The enzymatic activity (units/20 seeds) of α-galactosidase I and α-galactosidase II obtained after Sephadex G-150 gel filtration from different periods of germination was determined. The enzyme was assayed by the procedure described in 'Experimental Procedure'.


∝ – Galactosidase II
∝ – Galactosidase I
Enzyme activity ( units / 20 seeds )
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Germination period ( days )

activities of α-galactosidase I and α-galactosidase II were altered significantly. In dormant seeds the total activity of α-galactosidase I (50 units/20 seeds) was much higher than that of α-galactosidase II (3 units/ 20 seeds). But during germination the pattern changed entirely. After one day of germination only one-third activity of α-galactosidase I was formed. However, there was a marked increase in the total activity of α-galactosidase II, representing both α-galactosidase IIa and IIb. Following this, α-galactosidase I showed a marked decline in its activity. But the α-galactosidase II increased regularly during the germination reaching a maximum level on 6th day of germination. At this stage the ratio of α-galactosidase II to α-galactosidase I activity was about 18 compared to 0.06 in the dormant seeds. On 7th day of germination both the levels of α-galactosidase I and α-galactosidase IIa started to decline.

## 4.9 EFFECT OF CYCLOHEXIMIDE ON THE BIOSYNTHESIS OF DIFFERENT α-GALACTOSIDASE ISOENZYMES

Cycloheximide is a potent inhibitor of protein synthesis on 80S ribosomes in plants (20) . The effect of cycloheximide on the biosynthesis of α-galactosidase has been investigated by determining the levels of various isoenzymes during a specified period of germination. For this purpose nearly 20 germinating cotyledons

were at randomly picked up and α-galactosidase activity was determined. The data, summarized in Fig 24 show the effect of cycloheximide on the synthesis of total α-galactosidase at different stages of germination. Since, cycloheximide was applied only after 12 hours of imbibition period, the level of α-galactosidase enzyme at this stage was taken as 100% . During this period 90 units/20 cotyledons were synthesized in the untreated seeds. After 2 days there was a net synthesis of 20 units in control whereas in the treated ones showed a loss of 25 units during this period. Thereby an inhibition of 40.9% occured. After 4 days and 6 days of treatment 38.46% and 23.53% inhibition occurred, respectively.

In order to find out the effect of cycloheximide on the synthesis of various α-galactosidase species, the α-galactosidase isoenzymes were separated by Sephadex gel filtration on the basis of molecular weight. Fig.25 shows the elution profiles of high and low molecular weight α-galactosidase isoenzymes at different stages of germination in the presence of cycloheximide. The α-galactosidase isoenzyme patterns of cycloheximide treated seeds were same as that of untreated seeds upto second day of germination. Second day onwards the patterns start to revert to the dormant seed pattern with an increase in the activity of high molecular weight form and inhibition of synthesis of low molecular weight α-galactosidase by cycloheximide treatment (Fig 26).

FIG. 24. EFFECT OF CYCLOHEXIMIDE ON α-GALACTOSIDASE ACTIVITY DURING GERMINATION.

Total units/20 seeds of α-galactosidase in cycloheximide treated (treatment was given after 12 h of imbibition in water for 6 h) and untreated black variety chickpea seeds were determined. The enzyme activity was determined as described in 'Experimental procedure'.

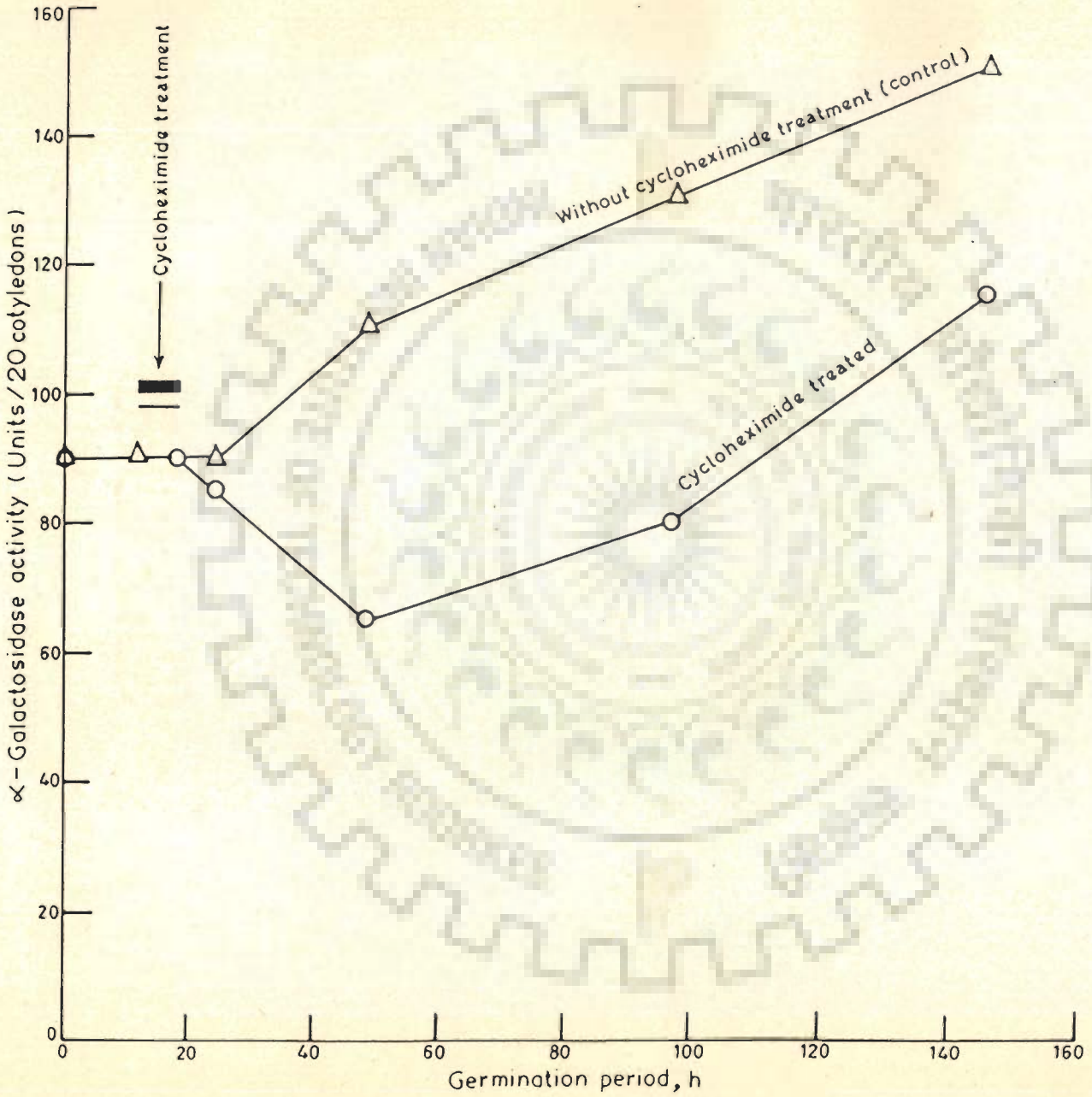

160
140
120
100
80
60
40
20
0
Cycloheximide treatment
Without cycloheximide treatment (control)
Cycloheximide treated
∝-Galactosidase activity (Units/20 cotyledons)
0
20
40
60
80
100
120
140
160
Germination period, h

FIG.25. SEPHADEX G-150 GEL FILTRATION PATTERNS OF α-GALACTOSIDASE ACTIVITY IN GERMINATING CHICKPEA SEEDS IN PRESENCE OF CYCLOHEXIMIDE.

The black variety chickpea seeds were soaked in water for 12 h, treated with 50 μmol solution of cycloheximidefor 6 h and then allowed to germinate in dark, the enzyme was extracted after different periods of germination and analyzed by Sephadex G-150 gel filtration as described in 'Experimental Procedure'.

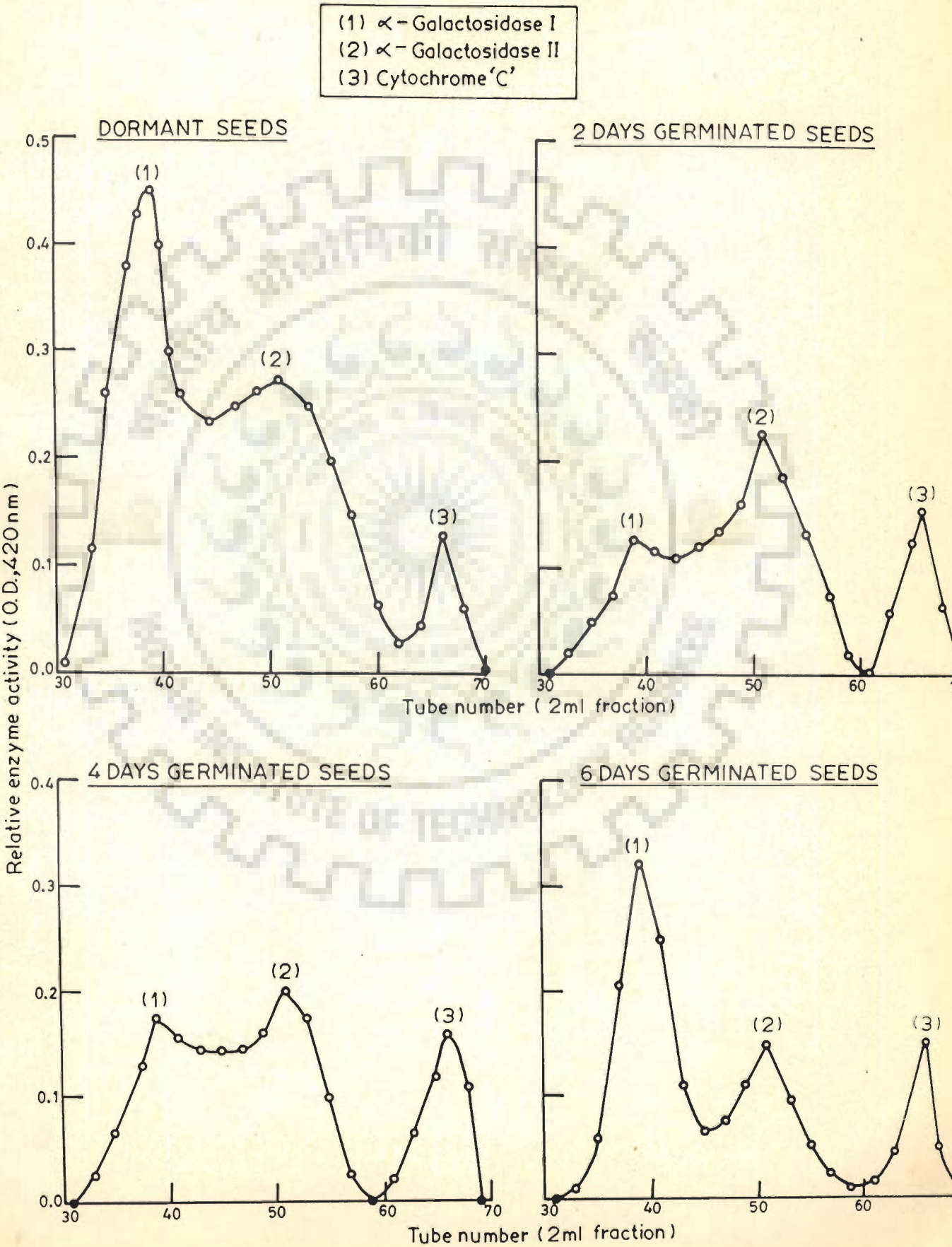
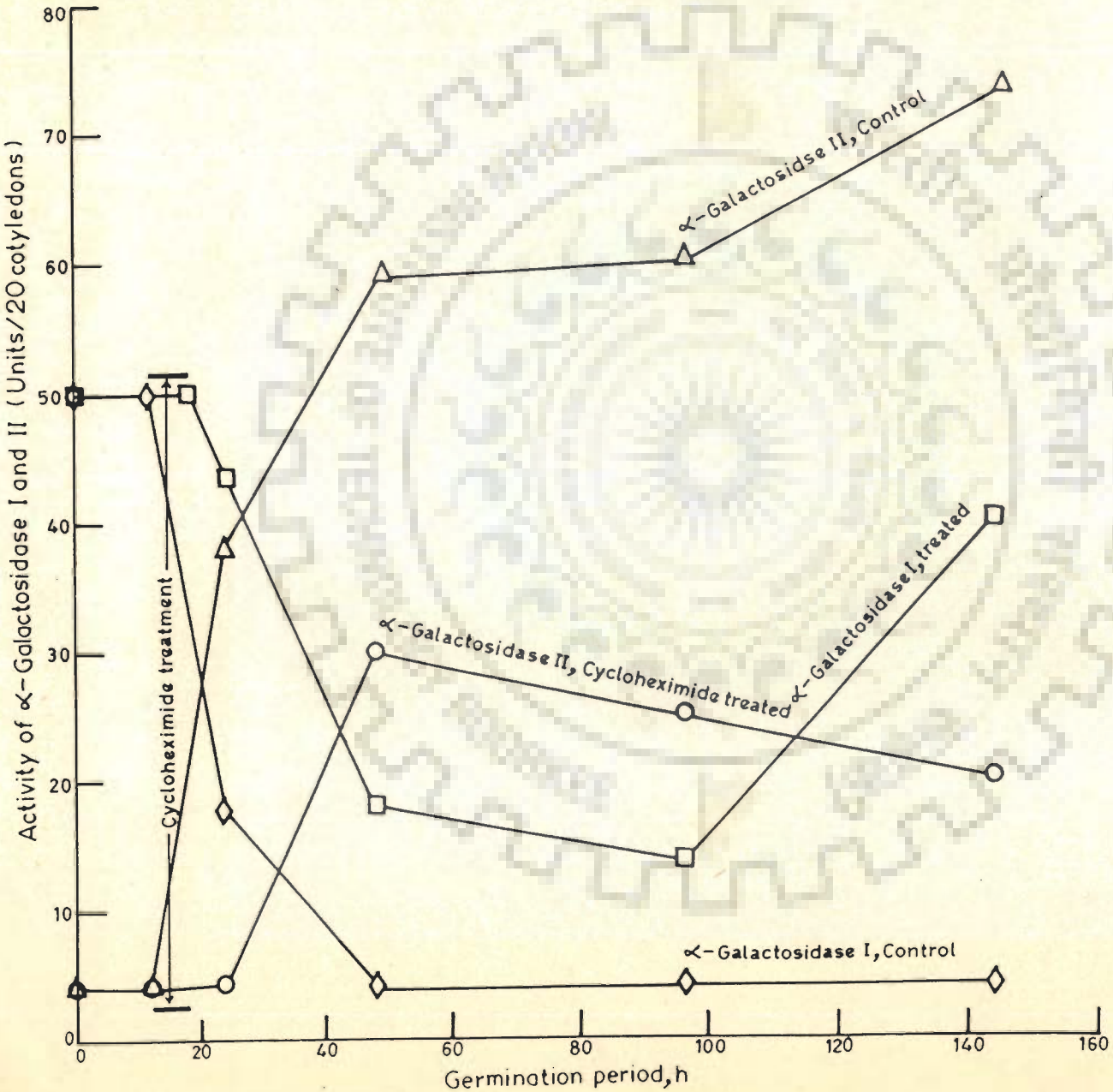

(1) ∝ - Galactosidase I
(2) ∝ - Galactosidase II
(3) Cytochrome 'C'
DORMANT SEEDS
2 DAYS GERMINATED SEEDS
4 DAYS GERMINATED SEEDS
6 DAYS GERMINATED SEEDS
Relative enzyme activity (O.D.,420nm)
Tube number ( 2ml fraction)
0.0
0.1
0.2
0.3
0.4
0.5
30
40
50
60
70

FIG.26. CHANGES IN THE LEVELS OF α-GALACTOSIDASE I AND α-GALACTOSIDASE II DURING GERMINATION IN PRESENCE OF CYCLOHEXIMIDE.

The enzymatic activity (units/20 seeds) of α-galactosidase I and α-galactosidase II obtained after Sephadex G-150 gel filtration after different periods of germination of cycloheximide treated seeds was determined by the method described in 'Experimental Procedure'.


Activity of ∝-Galactosidase I and II (Units/20 cotyledons)
Germination period, h
∝-Galactosidase II, Control
∝-Galactosidase II, Cycloheximide treated
∝-Galactosidase I, treated
∝-Galactosidase I, Control
Cycloheximide treatment
0
10
20
30
40
50
60
70
80
0
20
40
60
80
100
120
140
160

## 4.10 DEVELOPMENTAL CHANGES OF α-GALACTOSIDASE ISOENZYMES OF CHICKPEAS DURING SEED MATURATION

Samples of immature black variety chickpea seeds, collected at different stages of maturation, were examined for α-galactosidase activity. Fig 27 shows the elution profiles of α-galactosidase isoenzymes from Sephadex G-150 gel filtration chromatography. Examination of these results showed that in the very early stage of seed development (10 days after flowering), only α-galactosidase II was dominated with a very small peak of α-galactosidase I, indicating that probably the enzyme II was the first of the two molecular forms to be synthesized. After 17 days of flowering α-galactosidase I made its appearance and its level increased with the maturation of chickpea seeds. As the development of seed proceeded the level of total α-galactosidase activity also increased. In fact, in about 40 days time, when the seeds appeared quite mature, the activity of α-galactosidase I was significantly higher than that of α-galactosidase II. The results of total α-galactosidase activity and relative levels of α-galactosidase I and II are shown in Fig.28. In dormant seeds the level of enzyme II was very low (only 100 units/100 embroys) while α-galactosidase I was dominant (1499 units/100 embroys).

FIG.27. SEPHADEX G-150 GEL FILTRATION PATTERNS OF α-GALACTOSIDASE ACTIVITY OF BLACK VARIETY CHICKPEA SEEDS DURING MATURATION.

0.3 - 0.7 ammonium sulphate fractions in 0.05M sodium acetate buffer, pH 5.2, obtained from different periods of seed maturation of black variety chickpeas analyzed by gel filtration on Sephadex. Aliquots (0.1 ml) from every other fractions were tested for enzymatic activity as described in 'Experimental procedure'.

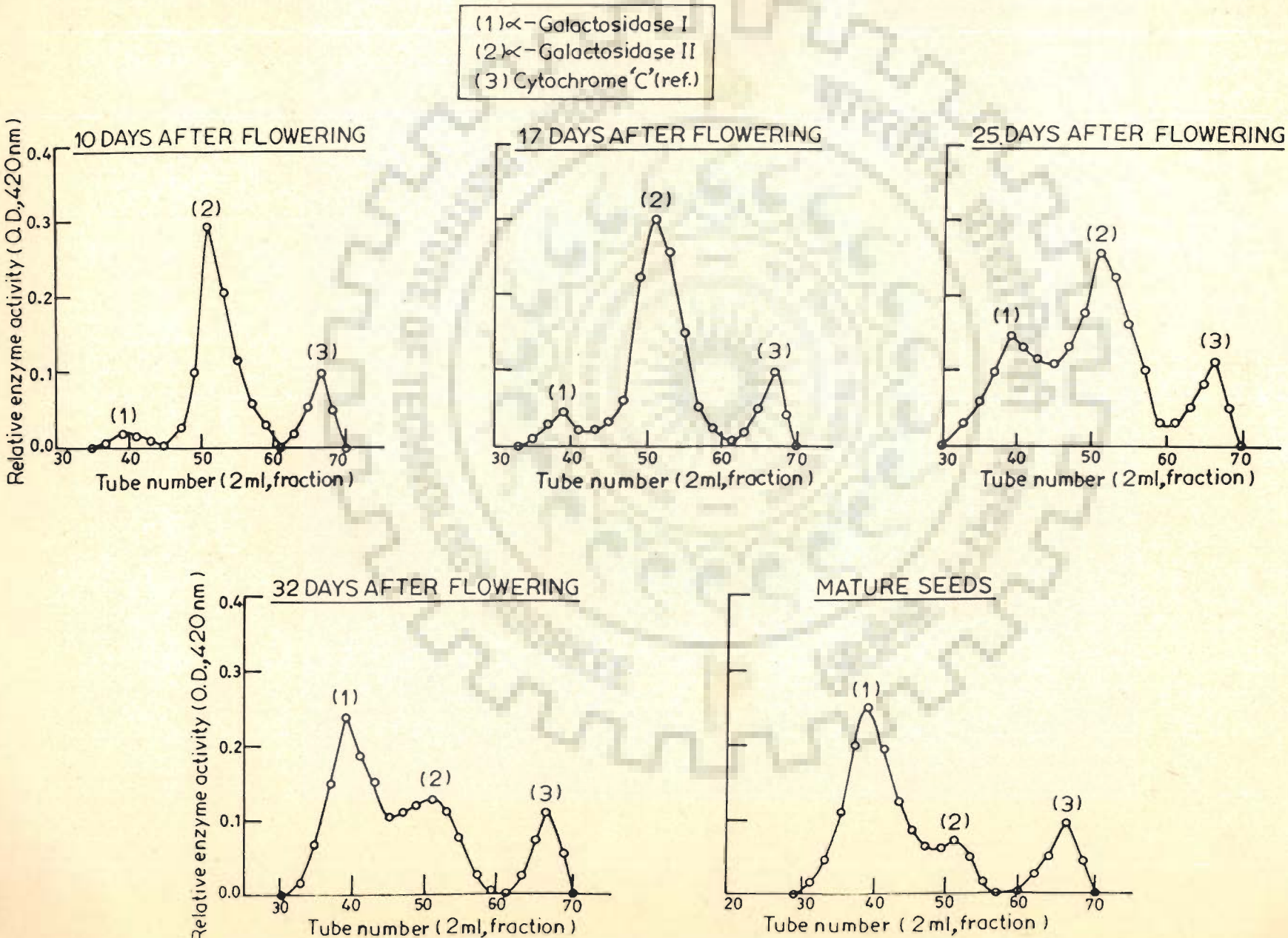
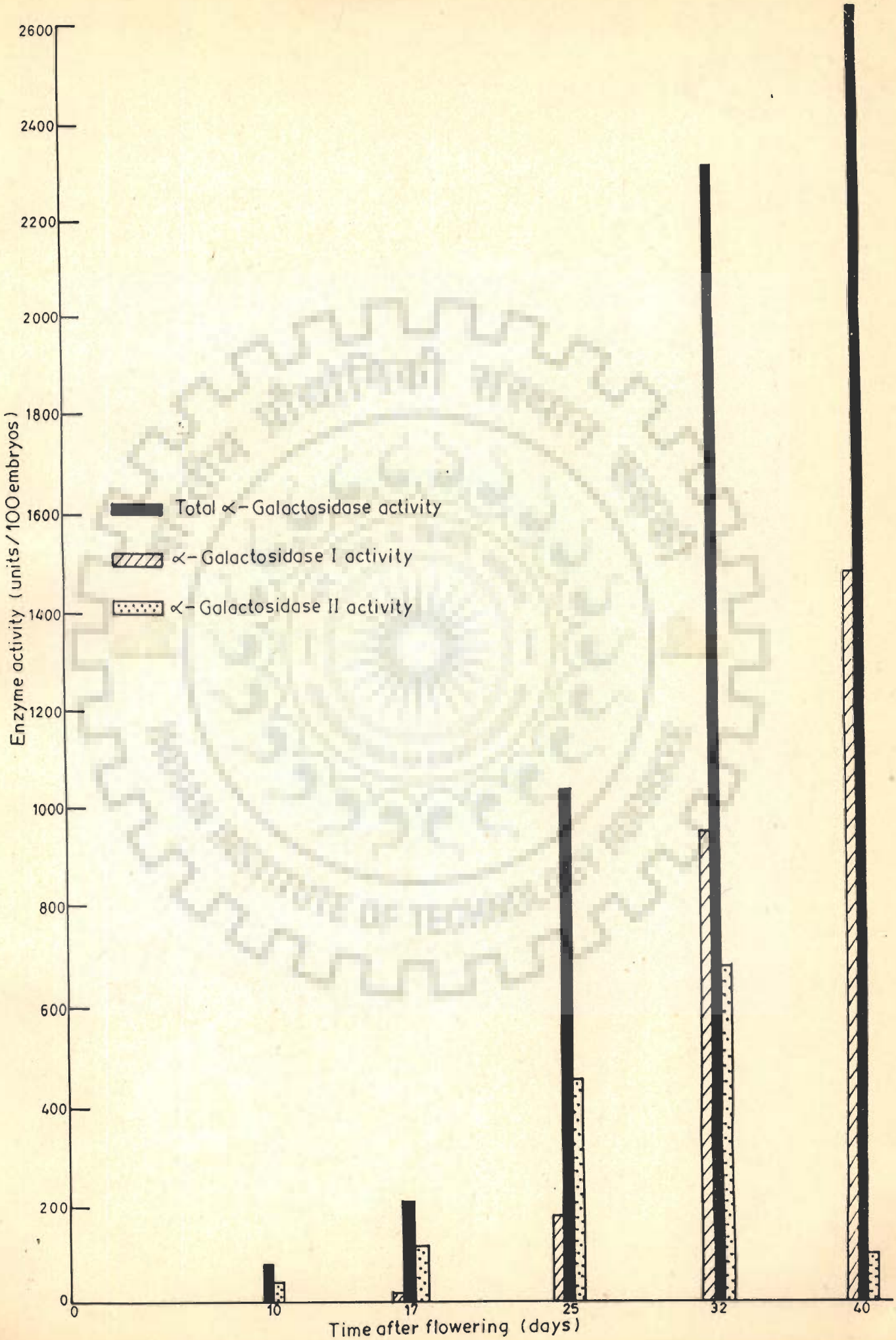

(1) ∝-Galactosidase I
(2) ∝-Galactosidase II
(3) Cytochrome 'C' (ref.)
10 DAYS AFTER FLOWERING
17 DAYS AFTER FLOWERING
25 DAYS AFTER FLOWERING
32 DAYS AFTER FLOWERING
MATURE SEEDS
Relative enzyme activity (O.D.,420nm)
Tube number (2ml, fraction)
0.0
0.1
0.2
0.3
0.4
20
30
40
50
60
70
(1)
(2)
(3)

FIG.28. RELATIVE CHANGES IN α-GALACTOSIDASE I AND α-GALACTOSIDASE II ACTIVITIES DURING MATURATION OF BLACK VARIETY CHICKPEA SEEDS.

The low and high molecular forms of α-galactosidase during the embryo development and seed maturation were separated by Sephadex gel filtration on Sephadex G-150. The relative level of various α-galactosidase form were compared.


Enzyme activity (units/100 embryos)
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Total ∝-Galactosidase activity
∝-Galactosidase I activity
∝-Galactosidase II activity
0
10
17
25
32
40
Time after flowering (days)

The increase in total enzymatic activity resembled the increase in total protein content of the cotyledons of developing chickpea seeds (Fig 29). The increase in glycosidases activity and total protein contents of the cotyledons of developing seeds had also been reported by Storey and Beevers, 1977 (171) and Neeley and Beevers in , 1980 (128). Deposition of reserve proteins can be detected after 17 days postanthesis and occurs rapidly between the period of 25 and 40 days. During this period the weight of embryos also increased markedly.

## 4.11 ISOENZYMIC PATTERN OF α-GALACTOSIDASE ISOENZYMES IN DIFFERENT VARIETIES OF CICER-ARIENTINUM SEEDS

The isoenzymic patterns of α-galactosidase isoenzymes in different varieties of Cicer-arietinum are shown in Table XVI. From these results it is clear that all the black varieties ($C_{255}$ (1,2,3,4,5), $H_{208}$ (1,2,3) local varieties 1,2,3,4,5,6,7,8 ) and white varieties (1K, 2K, 3K, 4K) differ from each other in the electrophoretic patterns of α-galactosidase isoenzymes and proteins in general in terms of number and intensity of protein . The black varieties of chickpea seeds showed a characteristic protein band (Fig 30) Mr = 149,600 dalton (band a) indicating the presence of high molecular weight α-galactosidase I. This band

FIG.29. CHANGES IN THE LEVEL OF EMBRYONIC WEIGHT AND AMOUNT OF PROTEIN DURING MATURATION OF CHICKPEA SEEDS.

Fresh weight of 100 embryos was determined at different stages of seed maturation. The protein was estimated in the crude homogenate at each stage by 'Lowry Method'.

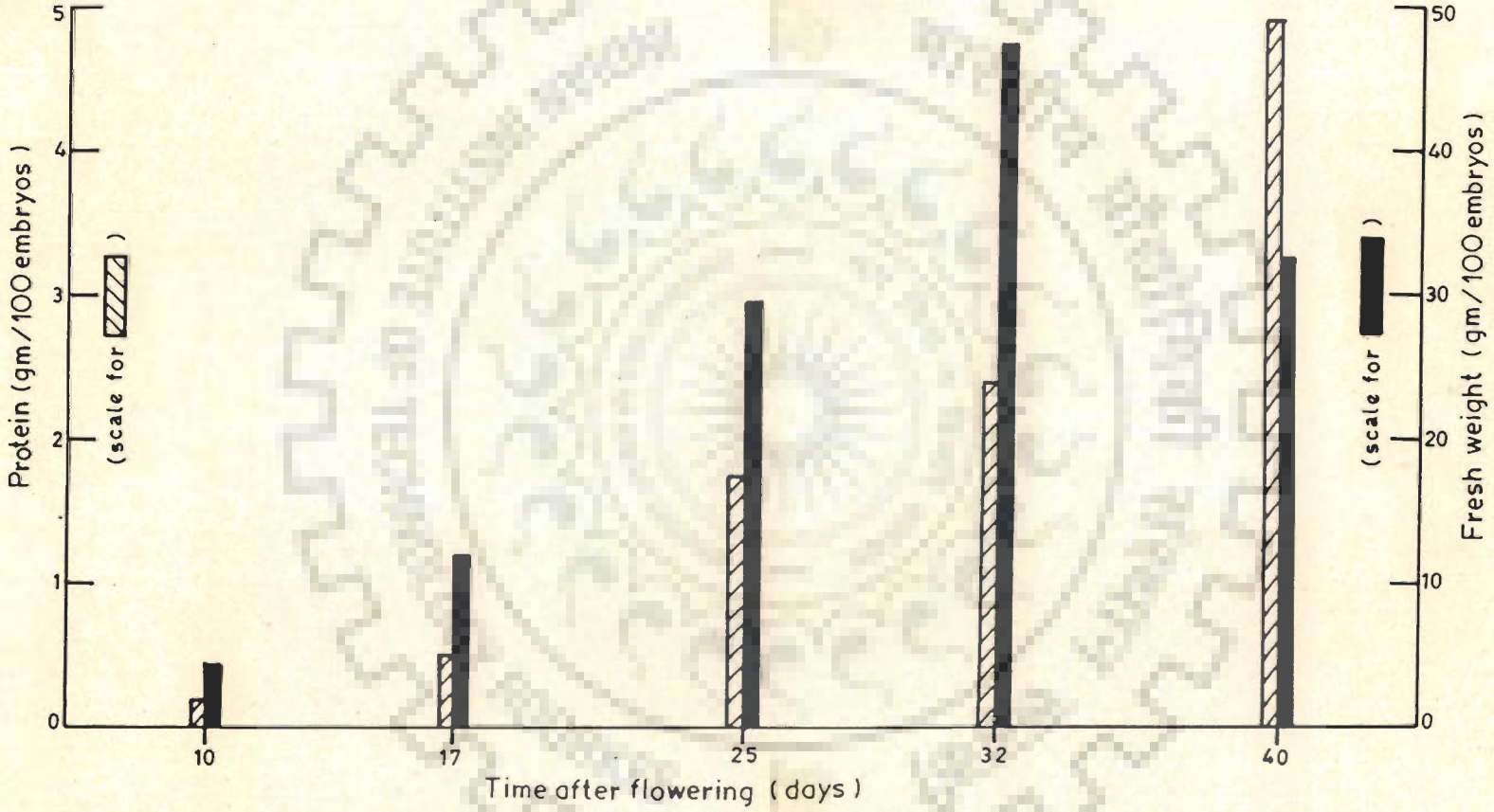

Protein (gm/100 embryos)
(scale for
)
Fresh weight (gm/100 embryos)
(scale for
)
0
1
2
3
4
5
10
20
30
40
50
10
17
25
32
40
Time after flowering (days)

FIG. 30. ELECTROPHORETIC PATTERN OF PROTEINS IN DIFFERENT VARIETIES OF CICER-ARIETINUM SEEDS.

The SDS polyacrylamide gel electrophoresis of 0.3 to 0.7 $(NH_4)_2 SO_4$ fraction followed by dialysis as described in the text of each variety was done. The electrophoresis was carried out with 7.5% gels by the method described in 'Experimental Procedure'. 200 µg of protein from each sample was loaded on separate gel columns. The direction of electrophoresis was from top to bottom.

# ELECTROPHORETIC PATTERN OF PROTEINS IN DIFFERENT VARIETIES OF CICER ARIETINUM SEEDS


$C_{235(1,2,3,4,5)}$, $H_{208(1,2,3)}$, 1,2,3,4,5,6,7,8 – BLACK VARIETIES

1K, 2K, 3K, 4K – WHITE VARIETIES

←a, b, c, d – ∝-GALACTOSIDASE ISOENZYMES

was absent in all white varieties (1K, 2K, 3K, 4K) of chickpea seeds. Further, a remarkable difference in white and black variety of seeds was the presence of a low molecular weight α-galactosidase isoenzyme of Mr=25,000 daltons (protein d) in all white varieties of seeds which was absent in black varieties seeds. The relative intensity of these bands also varies from variety to variety (Table XVI).

In order to find whether the high molecular weight α-galactosidase I was absent in white varieties at all stages of development or appear at some stage, the samples of white variety seeds were collected at different stages of development and analyzed for α-galactosidase isoenzymic pattern by Sephadex G-150 gel filtration. Fig 31 and 32 show the elution profiles of α-galactosidase isoenzymes during germination and maturation. These results clearly indicate that α-galactosidase I did not appear at any stage of development of white varieties. However, a very low molecular weight referred to a α-galactosidase III was found to exist at all stages of germination and maturation in white variety seeds. This low molecular weight enzyme was absent from the seeds of black varieties. The physiological significance of these observation must wait further investigation.

TABLE XVI α-GALACTOSIDASE ISOENZYMES IN DIFFERENT VARIETIES OF CICER-ARIETINUM SEEDS

| Variety | α-galactosidase Isoenzymes | | | |
|---|---|---|---|---|
| | I (Mr=149,000)* | IIa (Mr=56,000)* | IIb (Mr=50,000)* | III (Mr=25,000)* |
| **Black varieties** | | | | |
| $C_{235}(1)$ | + [a] | + | +++ | - [b] |
| $C_{235}(2)$ | + | + | ++ | + |
| $C_{235}(3)$ | + | - | - | ++ |
| $C_{235}(4)$ | + | - | - | - |
| $C_{235}(5)$ | + | ++ | - | - |
| $H_{208}(1)$ | + | ++ | +++ | ++ |
| $H_{208}(2)$ | + | - | - | - |
| $H_{208}(3)$ | ++ | - | ++ | + |
| 1 | + | ++ | - | + |
| 2 | + | - | ++ | ++ |
| 3 | ++ | - | + | - |
| 4 | + | - | +++ | - |
| 5 | + | + | +++ | + |
| 6 | + | ++ | +++ | - |
| 7 | + | - | - | - |
| 8 | + | - | ++ | - |
| **White varieties** | | | | |
| 1K | - | + | ++ | + |
| 2K | - | - | - | ++ |
| 3K | - | - | +++ | ++ |
| 4K | - | + | - | ++ |

Crude extract from different varieties of chickpea was analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods.

a) +, ++, +++ show relative intensities of bands corresponding α-galactosidase I, IIa, IIb and III.

b) -, Absence of protein band of corresponding α-galactosidases

* daltons

FIG. 31. SEPHADEX G-150 GEL FILTRATION PATTERNS OF α-GALACTOSIDASE ACTIVITY OF WHITE VARIETY CHICKPEA SEEDS DURING GERMINATION.

0.3 to 0.7 ammonium sulphate fractions in 0.05 M sodium acetate buffer, pH 5.2, obtained from different seeds were analyzed on a Sephadex G-150 column (1X80 cm) at a flow rate of 0.1 ml/min . Aliquots (0.1 ml) from every other fractions were tested for enzymatic activity as described in 'Experimental Procedure'.

(1) ∝-Galactosidase II
(2) ∝-Galactosidase III
(3) Cytochrome 'C' (ref.)

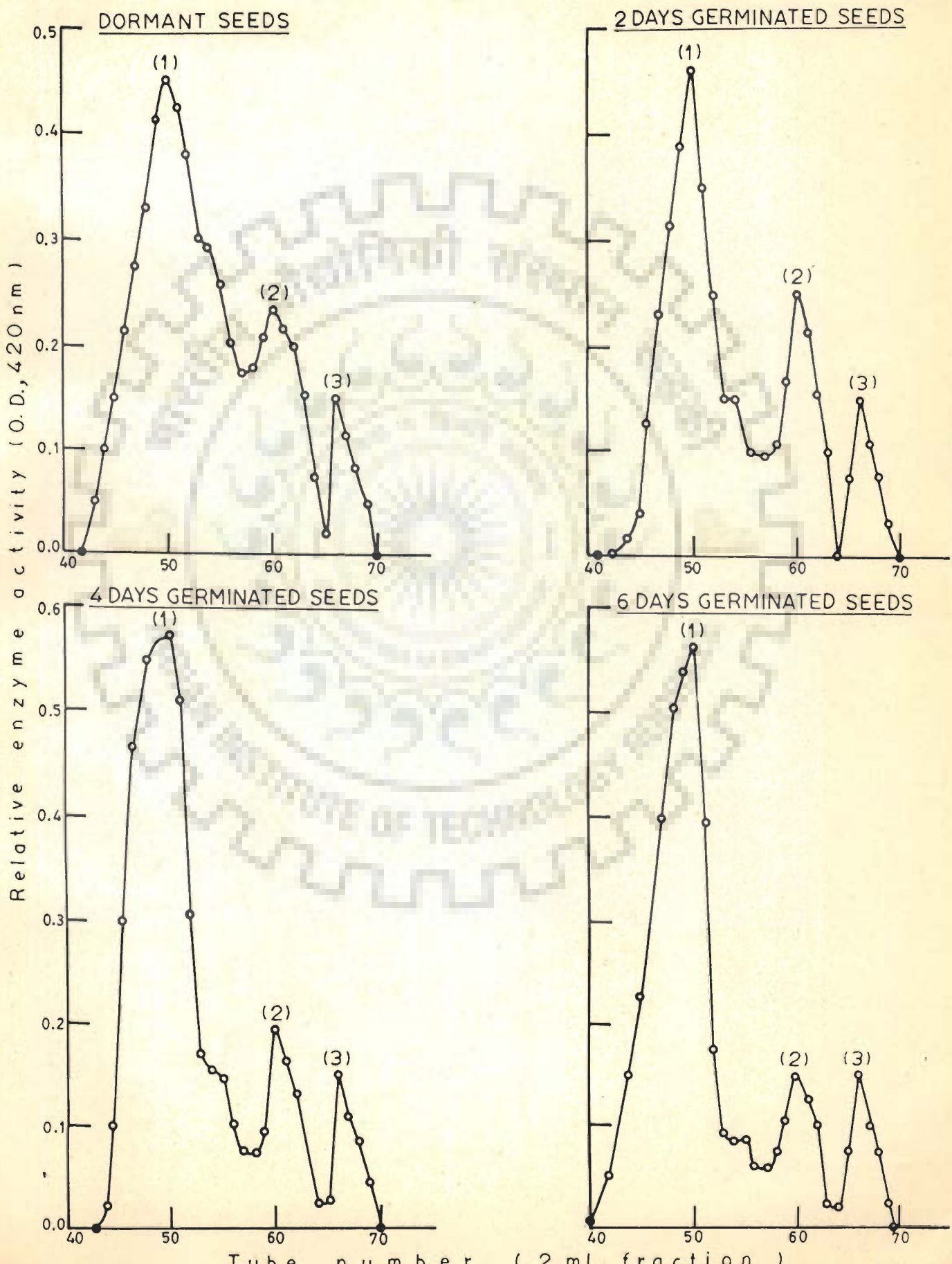
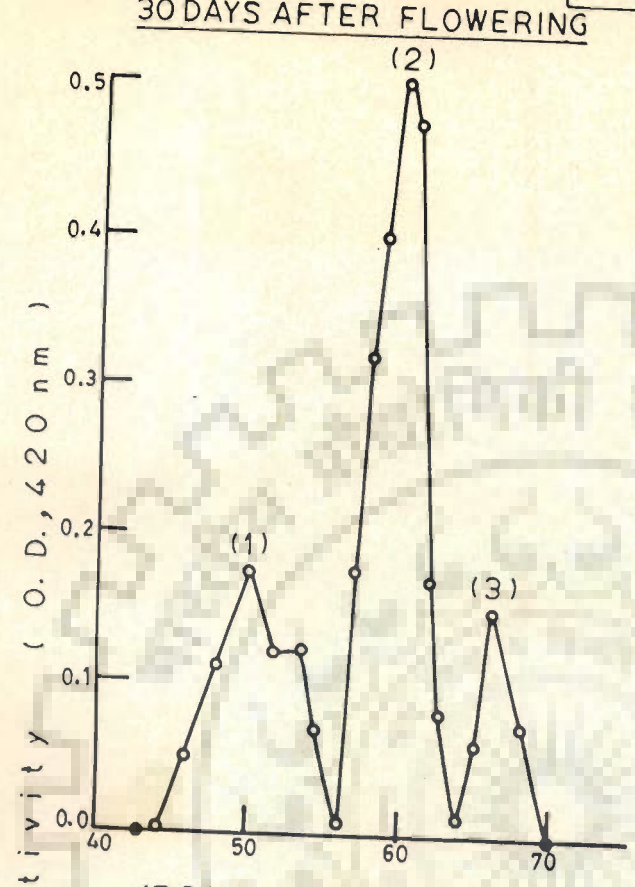
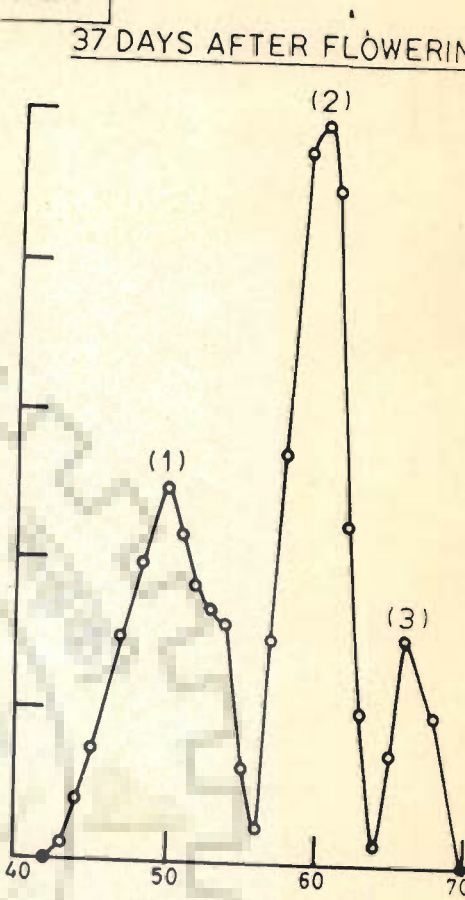


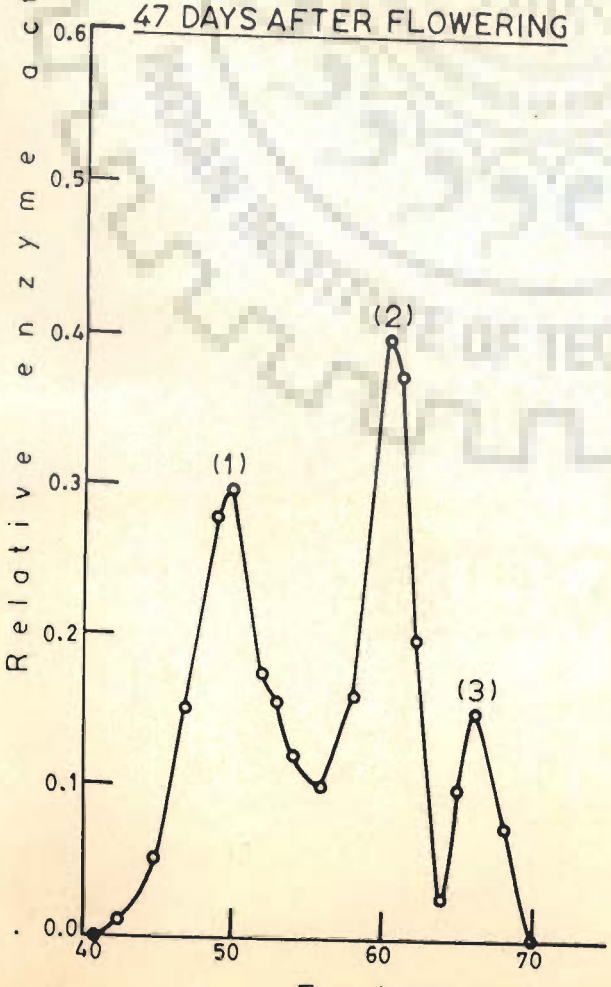
FIG. 32. SEPHADEX G-150 GEL FILTRATION PATTERNS OF GALACTOSIDASE ACTIVITY OF WHITE VARIETY CHICKPEA SEEDS DURING MATURATION.

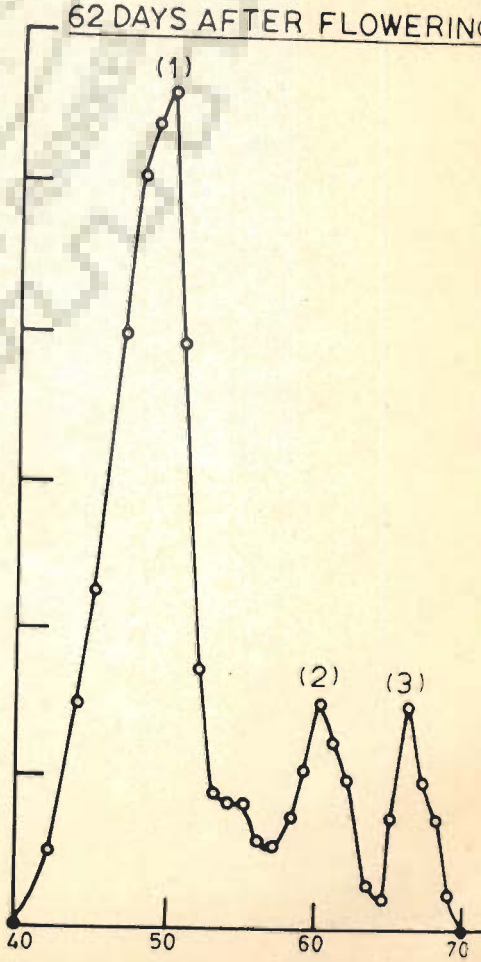
0.3 to 0.7 ammonium sulphate fractions in 0.05M sodium acetate buffer, pH 5.2, obtained from different stages of maturation of white variety chickpea seeds were filtered on a Sephadex G-150 column (1X80cm) at a flow rate of 0.1 ml/min . Aliquots (0.1 ml) from every other fractions were tested for enzyme activity.

(1) ∝- Galactosidase II
(2) ∝- Galactosidase III
(3) Cytochrome 'C' (ref.)



## 5.0 DISCUSSION

Chickpea is an important pulse crop of India and is available in many different varieties which are easily distinguishable by their distinct characteristic facial features. Like most other leguminous seeds (51,62,80) chickpeas were also found to be an excellent source of α-galactosidase and also to contain different forms of the enzyme. For the facts that chickpea is a nutritionally and commercially important pulse seed, exists in many varieties and contained multiple forms of α-galactosidase, make it an interesting organism for studying the biochemistry of α-galactosidases in seeds.

The results described here indicated that 6-days old germinating cotyledons provided the most suitable source for the isolation of multiple forms of α-galactosidase as at this physiological stage total as well as the specific activity of α-galactosidase was maximal, indicating a state of high metabolic activity in germinating cotyledons. In addition, the polysaccharide gums, normally present in all leguminous seeds, were removed during germination. Otherwise presence of such gums greatly interfere in subsequent steps of purification.

Using a six step purification scheme (Fig 3) it was possible to separate and purify three isoenzymes of α-galactosidase to electrophoretic homogeneity with

reasonably fair yields. The main features of the purification scheme were acetone treatment, pH precipitation, ammonium sulphate fractionation, gel permeation and ion exchange chromatography. The first three steps formed the initial treatment and provided a 2.8 folds purification with about 57.6% yield. Examination of the crude extract and of the ammonium sulphate fraction by gel filtration on Sephadex G-150 gave comparable elution profiles of α-galactosidase isoenzymes indicating that all the isoenzymes were conserved during these initial steps. Otherwise the conditions of enzyme extraction, etc. often influence the isoenzymic pattern (29,59,76,85). By gel filtration two molecular species, high molecular weight (α-galactosidase I, Mr=149,600) and low molecular weight (α-galactosidase II, Mr=56,000 ± 6000) were obtained. The later form of α-galactosidase was predominant with nearly three times higher specific activity than the former, suggesting a more major role of α-galactosidase II in the germinating cotyledons. Hence, α-galactosidase was purified further. A close look of α-galactosidase II peak from the Sephadex G-150 column showed that the peak was not quite symmetrical (Fig 4) since a shoulder in the peak could be seen at an elution volume of 106-108 ml. These results suggest that there may be present two α-galactosidase species having only slightly different molecular weights. Our attempts to separate the two forms of α-galactosidase II

by Sephadex G-75 or G-100 gel filtration did not succeed. The DEAE-cellulose column chromatography also did not work. Infact, almost 90% of the total enzyme activity was lost on DEAE-cellulose column chromatography. But there was some indication of the presence of α-galactosidase II isoenzymes differing in their ionic behaviour (Fig 5). It was later found that chickpea α-galactosidase was highly unstable in alkaline medium. We, therefore, used carboxymethyl cellulose chromatography at pH 5.2 as was used by Dey and Pridham (50). Two distinct peaks containing α-galactosidase activity (α-galactosidase IIa and α-galactosidase IIb) were obtained at elution gradients of 240 mM and 345 mM Nacl, respectively. In this regard the chickpea α-galactosidase showed identical behaviour to that of Vicia faba seeds (50).

The molecular weights of two forms of α-galactosidase II, measured by a carefully monitored Sephadex G-150 column were found to be 56,000 and 50,000 daltons, respectively. Thus, results of Sephadex column were confirmed and the isoenzymic pattern of crude extract seems to have been conserved through out the purification steps. Although, possibility of some interconversion to influence the relative concentrations of each form of α-galactosidase cannot be ruled out completely, because variations in the number of molecular species under different salt conditions, pH, etc., have been observed in several cases (29,43,44,59).

In the present purification scheme, however, the pH of the buffer system used was maintained constant at pH 5.2 ± 0.1, thereby greatly minimizing the possibility of producing varying patterns of multiple forms of α-galactosidase. It may also be pointed out that the relative concentration of α-galactosidase isoenzymes, as evident from the elution profiles of Sephadex G-150 (Fraction nos. 44-52 and 53-57), seems to be in near agreement with that of CM-cellulose pattern.

In summary, we believe that little or no change has occurred in the isoenzymic pattern of α-galactosidase during purification steps. This is an important aspect from the point of developmental studies where gel filtration and CM-cellulose chromatography have been employed for determining the relative concentrations of different molecular weight forms of α-galactosidase isoenzymes during seed germination and maturation.

The α-galactosidase IIa and α-galactosidase IIb were found to be pure by polyacrylamide gel electrophoresis giving only a single sharp band in each case. Both enzymes show different electrophoretic mobility confirming the different ionic character as shown by CM-cellulose column chromatography. The difference in ionic property appears to be true because of the fact that chromatography was carried out at an acidic pH while the electrophoresis was

carried out in alkaline medium. But on SDS gel electrophoresis under completely reduced conditions both α-galactosidase IIa and α-galactosidase IIb were splitted into three and two bands, respectively, indicating a subunit structure of the enzymes. Whether the subunits are distinct enzyme species or represent monomers, dimers and trimers, etc. is not certain at the present moment. Dey et al.(60) have reported similar results in the case of Vicia faba seeds where all three isoenzymes showed microheterogeneity by SDS polycrylamide gel electrophoresis but gave a single sharp band on polyacrylamide gel electrophoresis without SDS. Further studies will be required to establish the subunit structure of α-galactosidases.

Results presented here show that 6-days old germinated cotyledons contained three distinct species of α-galactosidase, viz., a high molecular weight form (α-galactosidase I, Mr = 149,600) and two low molecular weight form (α-galactosidase IIa, Mr = 56,000 and α-galactosidase IIb, Mr = 50,000). Whether these forms represent multimolecular forms containing the same monomer is not clear. However, on the basis of molecular weight it seems that the high molecular weight form is a trimer of the low molecular weight species. That it is likely to be so is supported by the fact that when the mixture of two low molecular weight forms of α-galactosidase are stored at pH 5.2 for sufficiently long period (40-70 days),

they associated to give a high molecular weight species which closely resembled the α-galactosidase I (Table XIV). It is interesting to note that neither α-galactosidase IIa nor α-galactosidase IIb when stored separately was converted to high molecular weight form of α-galactosidase. It was only when a mixture of α-galactosidase IIa and α-galactosidase IIb in the ratio of 3:1 was stored that the reconversion to high molecular weight form of α-galactosidase occurred. These results indicated the close structural relationship between the high and low molecular weight forms of α-galactosidase. The reconversion to the high molecular weight form of α-galactosidase from the low molecular weight forms has also been reported for several seeds by various workers (43,44,82). Dey, et al.(60,61) also demonstrated that a single subunit constituted the tetrameric glycoprotein enzyme. In the present case the α-galactosidase IIa and IIb which differs slightly in their apparent molecular weights are glycoproteins having widely different carbohydrate contents. For instance, the carbohydrate content of α-galactosidase IIb is nearly three times higher than that of α-galactosidase IIa. Whether the differences in properties in α-galactosidase IIa and IIb is due to the carbohydrate moiety is not clear. Similarly it is also not clear whether the subunit structure exhibited by both α-galactosidase IIa and IIb on SDS

polyacrylamide gel electrophoresis was due to the hetereogeneity of carbohydrate moiety of enzyme or to totally dissimilar subunits. The molecular weights of Cicer-arientinum α-galactosidase isoenzymes follow the pattern of multimolecular forms of α-galactosidase found in other seeds (62), although the molecular weights of α-galactosidase IIa and IIb are somewhat heigher giving a monomer/trimer relationship between the low molecular weight forms and the high molecular weight form of α-galactosidase instead of monomer/tetramer relationship shown by α-galactosidase of several seeds (60,77,79,80). In most cases reported so far the structural relationship between low and high molecular weight α-galactosidase species varies from dimeric to tetrameric.

Like many other legume α-galactosidases the chickpea α-galactosidase isoenzymes are glycoproteins (60,61,77). Since the accurate molecular weight determination of glycoproteins is difficult (104), the variation in the molecular weight may be expected. The glycoprotein moiety seems to be linked the asparagine residue of the polypeptide chain through N-glycosydic linkage, since the carbohydrate moiety was largely removed by treatment with N-acetyl-β-glucosaminidase (Endo-H). The qualitative analysis of monosaccharides showed the presence of N-acetyl glucosamine and mannose. Thus, like other glycoprotein glycosidases the glycon moiety in chickpea α-galactosidases appears to be of the high mannose type (54,61).

The molecular forms of Cicer-arietinum α-galactosidase exhibit both similarity and dissimilarity in properties. For instance, pH optimum, substrate specificity, inhibition by metal ions and sugars are similar except the degree of response towards various substrates and inhibitors. The different molecular forms showed markedly different thermodynamic parameters, electrophoretic mobility, carbohydrate content, $K_m$ and $V_{max}$ value.

Of the heavy metal ions tested only $Ag^+$ and $Hg^{2+}$ were found to be strong competitive inhibitors of chickpea α-galactosidases. The high sensitivity of both forms of α-galactosidase towards $Ag^+$ and $Hg^{2+}$ suggests the participation of thiol group (s) in the enzyme substrate reaction. The Pneumococcal α-galactosidase was found to be sensitive to $Hg^{2+}$ and sulphydral reagents (107). The nature of $Ag^+$ inhibition of α-galactosidase I, IIa and IIb was different from the nature of $Ag^+$ inhibition of coffee bean α-galactosidases which was non-competitive (115). In the latter case it was suggested by Malhotra and Singh (115) that in the light of carboxyl and imidazolium groups forming the part of the active sites in α-galactosidases the $Ag^+$ may interact with both these groups. The presence of positively charged metal ions in the vicinity will facilitate the deprotonation of the imidazolium group which, inturn will enhance the affinity of the enzyme for $Ag^+$ by coordination.

Generally α-galactosidases are inhibited competitively by free sugars (108,131,132), aldolactones (108) and polyols (163). However, in most cases the inhibitory action lacked the anomeric specificity since these compounds inhibited both α- and β- galactosidases. As reported by Sharma (162,163) D-xylose and myoinositol were steriospecific inhibitors of plant α-galactosidases. In agreement with these results we have also found that xylose and myinositol are powerful anomer-specific competitive inhibitor of chickpea α-galactosidase isoenzymes (Fig 33,34,35). But as expected, the degree of inhibition of different isoenzymes was different.

The selective inhibition of α-galactosidase by myoinositol was attributed to similarity in the orientation of - OH gps at $C_2$ , $C_3$ and $C_4$ in myoionositol molecule to those of $C_4$ , $C_3$ and $C_2$ or $C_1$ , $C_2$ and $C_3$ of α-galactopyranoside residue (Fig 36) of the substrate (162) Myoinositol being a competitive inhibitor, it is expected to interact directly on the active site of the enzyme. Thus, makes it suitable for use as an active site directed reagent for the determination of active site of different isoenzymes of α-galactosidase. Since the action of myoinositol is highly specific for α-galactosidase, it can be used in the presence of other glycosidases. The affinity ($1/k_i$) values of myoinositol towards different molecular forms of α-galactosidase were found to be

FIG. 33. DIXON PLOT FOR THE INTERACTION OF α-GALACTOSIDASE I WITH MYONINOSITOL.

The effect of varying concentrations of myoinositol (0-200 mM) on the rate of hydrolysis of P-nitrophenyl α-D-galactopyranoside by α-galactosidase I was determined at two different substrate concentrations (0.1 mM and 0.2 mM) in 0.05M sodium acetate buffer, pH 5.2 Reciprocal of velocity was plotted against inhibitor concentration and $K_i$ values were obtained from the intersection of the two curves as described in the text.

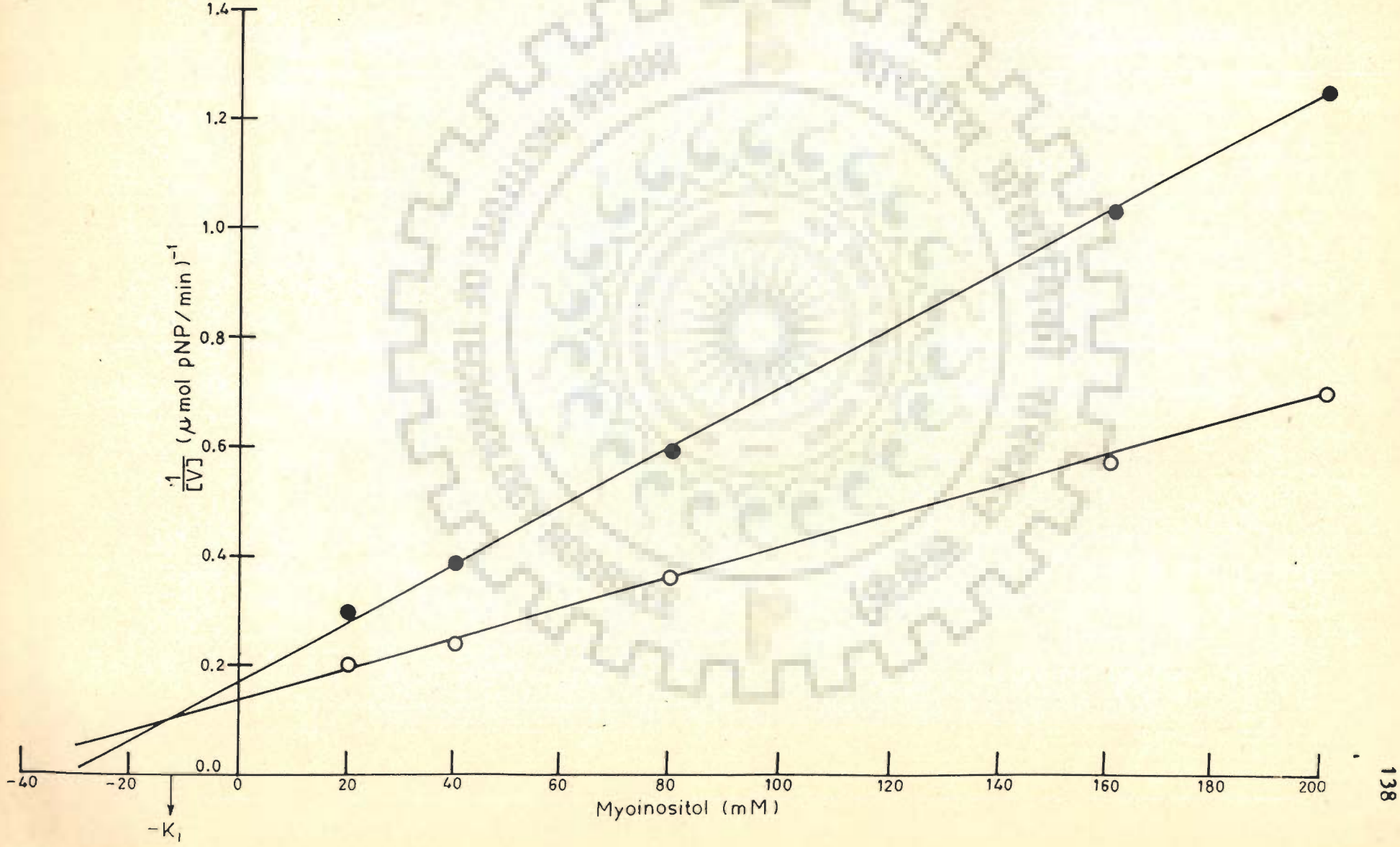

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
$\frac{1}{[V]}$ ($\mu$ mol pNP / min)$^{-1}$
-40
-20
0
20
40
60
80
100
120
140
160
180
200
Myoinositol (mM)
$-K_I$

FIG. 54. DIXON PLOTS FOR THE INTERACTION OF α-GALACTOSIDASE IIa WITH MYOINOSITOL.

The effect of varying concentrations of myoinositol (0-200 mM) on the rate of hydrolysis of p-nitrophenyl α-D-galactopyranoside by α-galactosidase IIa was determined at two different substrate concentrations (0.1 mM and 0.2 mM) in 0.05 M sodium acetate buffer, pH 5.2. Reciprocal of velocity was plotted against inhibitor concentration and $K_i$ values were obtained from the intersection of the two curves as described in the text.

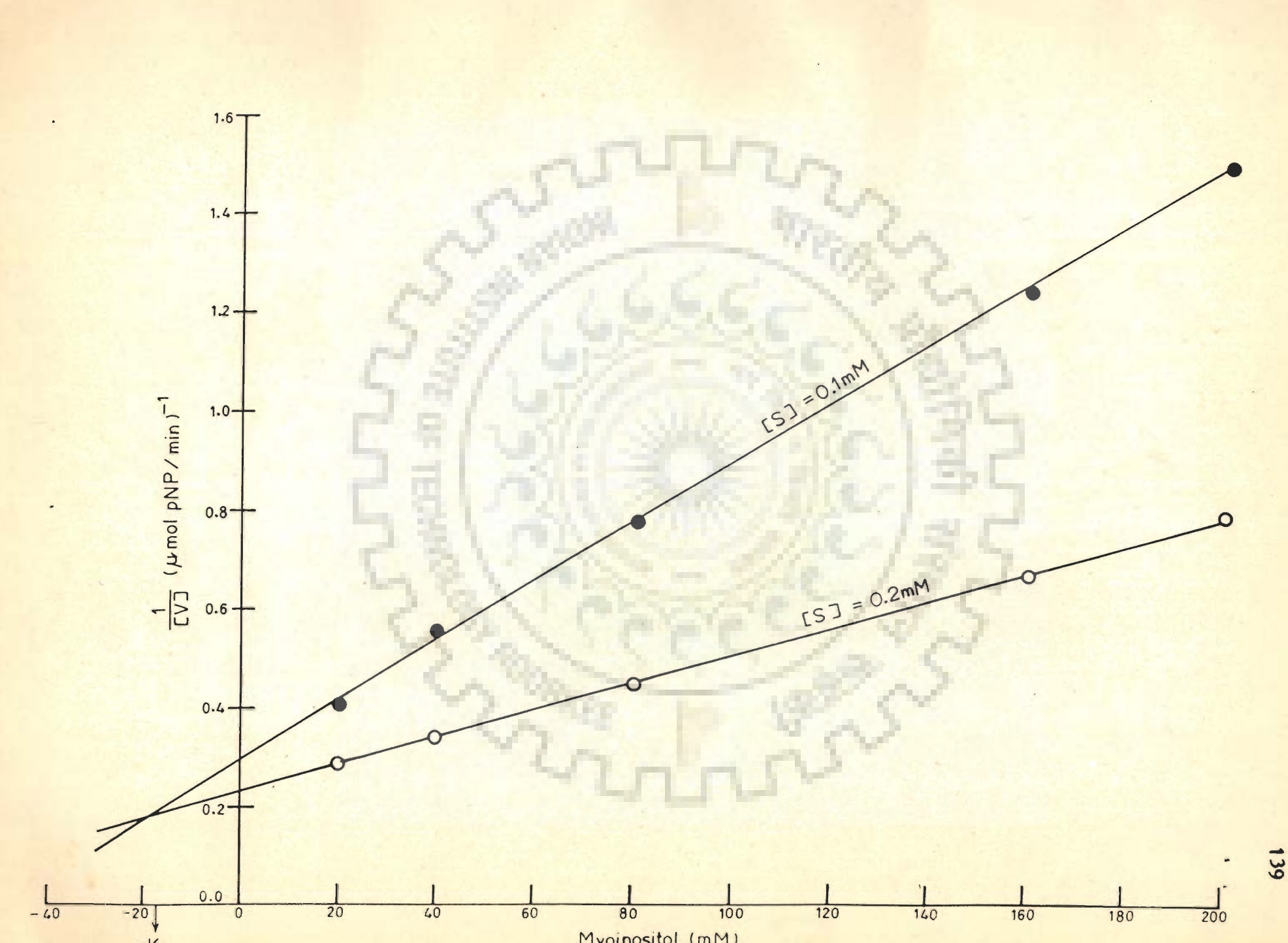
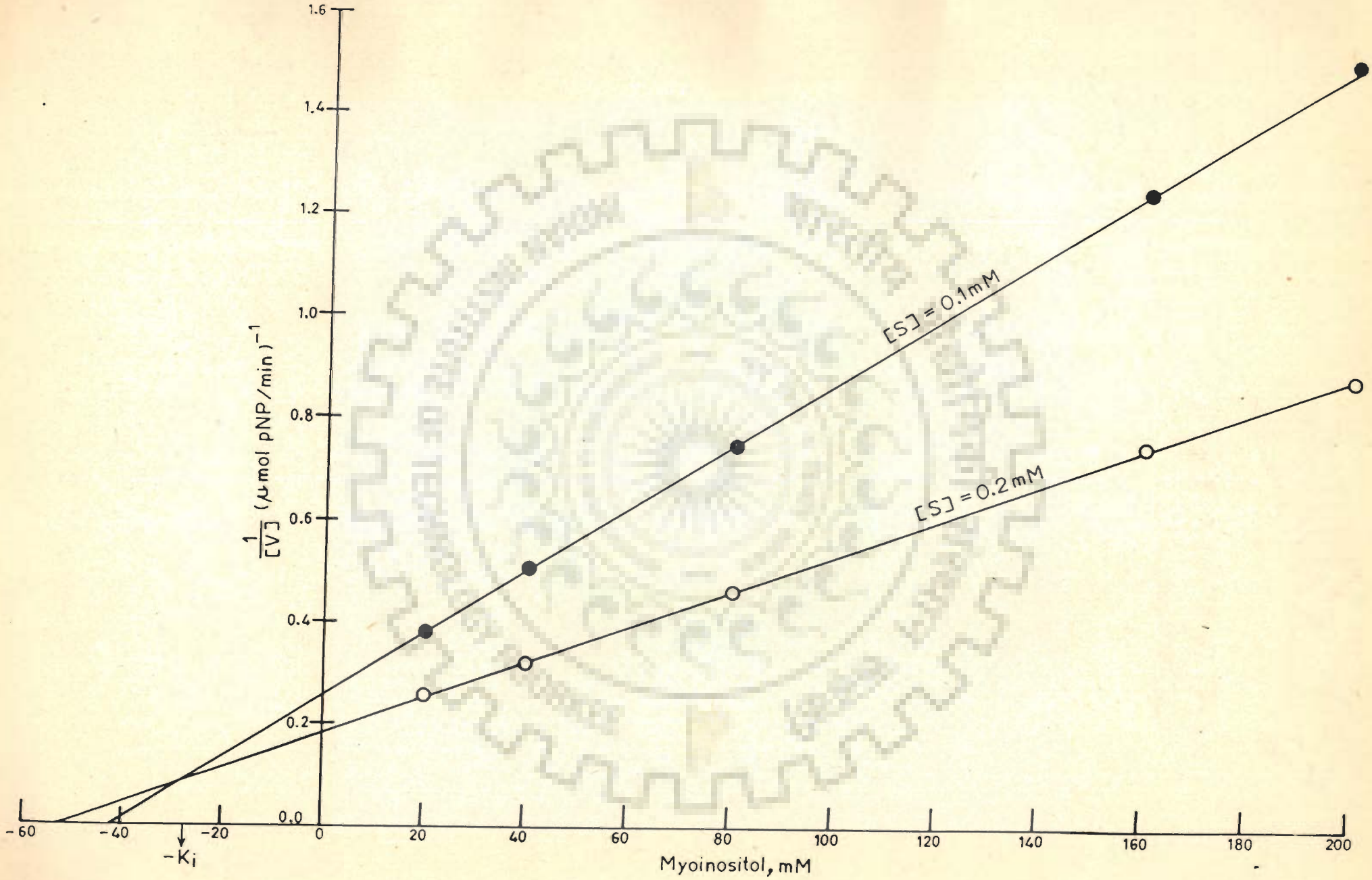
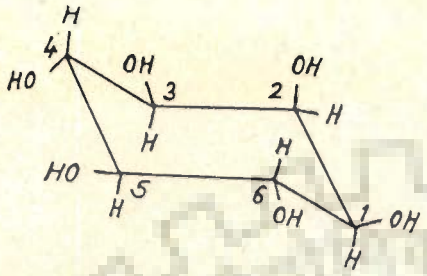

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1/[V] (μmol pNP/min)⁻¹
[S] =0.1mM
[S] = 0.2mM
-40
-20
0
20
40
60
80
100
120
140
160
180
200
Myoinositol (mM)

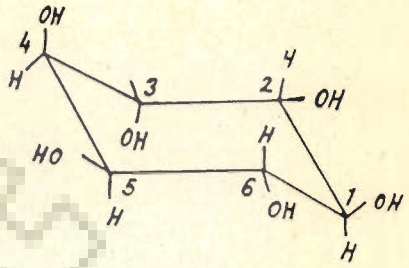
FIG.35 DIXON PLOTS FOR THE INTERACTION OF α-GALACTOSIDASE IIb WITH MYOINOSITOL.

The effect of varying concentrations of myoinositol (0-200mM) on the rate of hydrolysis of p-nitrophenyl α-D-galacto-pyranoside by galactosidase IIb was determined at two different substrate concentrations (0.1 mM and 0.2 mM) in 0.05 M sodium acetate buffer, pH 5.2. Reciprocal of velocity was plotted against inhibitor concentration and $K_i$ values were obtained from the intersection of the two curves as described in the text.

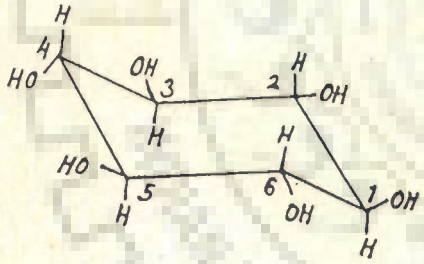




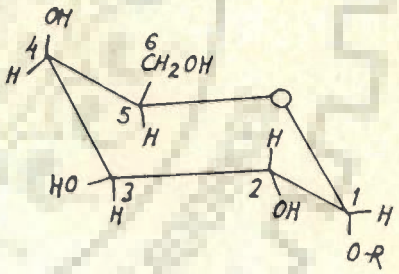
Myo-Inositol



(+) Inositol

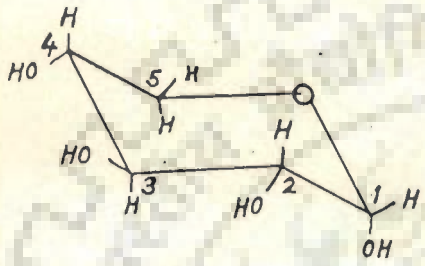


Scyllitol

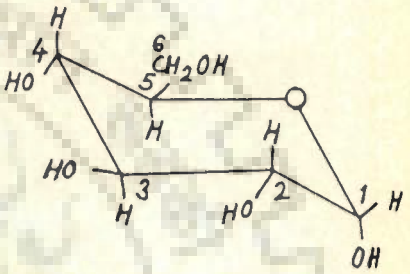


α-D-Galactoside
(R = p-Nitrophenyl)

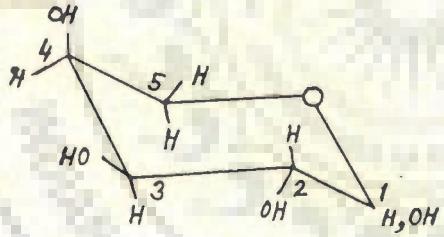
FIG. 36- Conformations of some sugars and sugar analogues.



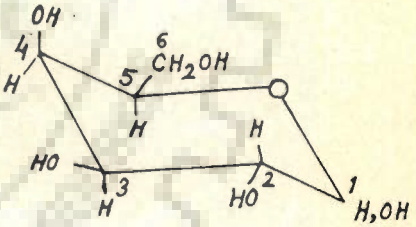

D-Xylose




D-Glucose




L-Arabinose




D-Galactose

Conformations of some sugars and sugar analogues.

markedly different. These results suggest, but by no means prove, that the active sites of high and low molecular forms of α-galactosidase possess different structures or conformations.

The immobilized enzyme in calcium alginate beads exhibited higher pH and thermal stability. This is of significance from the point of view of industrial application, since the immobilized α-galactosidase find application in food industries (35,113,145). The mechanism for the enhanced stability may be attributed to the selective environment surrounding the immobilized enzyme. The kinetics of the immobilized enzyme is also different from the free enzyme due to the difference in the over-all environment and partitioning of substrate between solution and the solid phase carrying the immobilized enzyme (25, 96). While measuring the kinetic parameters, such factors were taken into consideration. As expected the $K_m$ values of the immobilized enzymes were significantly higher than that of the free enzymes.

In order to investigate the physiological role and the structural relationship of α-galactosidase isoenzymes we have investigated the development of the enzyme during germination and maturation of the black variety (Local variety 1) chickpea seeds by Sephadex gel filtration. For all practical purpose we have assumed that α-galactosidase IIa and IIb represent a single molecular species. It

was found that during the process of germination the relative level of α-galactosidase I activity, which was predominant in dormant seeds, declined continuously with a concomitant increase of α-galactosidase II level. And finally on the 6th day of germination, when the metabolic activity was at its peak, the lower molecular weight α-galactosidase was highly predominant with only very small proportion of α-galactosidase I activity present. These results were interpretted to mean the following : (i) The synthesis of high molecular weight enzyme (α-galactosidase I) was stopped and, therefore, its level continuously decreased during germination, (ii) de novo synthesis of low molecular form (α-galactosidase II) occurred at a rapid rate, and (iii) α-galactosidase I was converted to low molecular weight α-galactosidase II.

Whether all or any of these events occur in regard to the development of α-galactosidase molecular forms during germination of chickpea seeds is far from clear. But it is quite certain that during the process of germination of chickpea seeds, the high molecular weight form (α-galactosidase I) was replaced by the low molecular weight form enzymes (α-galactosidase IIa and IIb).

The maturing chickpea seeds provided quite the opposite pattern in that the proportion of high molecular form enzyme increased continuously with concomitant

decrease in the level of the low molecular weight form enzyme. Thus, the developmental patterns of α-galactosidase species confirm the metabolic status of seeds, viz., during germination when reserve α-D-galactosyl-containing oligo-and polysaccharides are mobilized to meet the high energy requirement (51, 119, 139, 142, 159) the enzyme of low specific activity was replaced by the high specific activity enzyme form. On the other hand in the maturing seeds when the reserve carbohydrates are formed and deposited in storage tissues, the enzyme form with high specific activity was replaced by the low specific activity. In the latter case the trans-galactosylation activity might be operative *in vivo* as reported by Dey (55). From these results it can be concluded that relatively high dominence of the low molecular forms α-galactosidase over the high molecular form enzyme, and vice versa characterize the germinating and the maturing states of the chickpea seeds, respectively. And it appears likely that the over-all activity of α-galactosidase during the seed germination and maturation processes is regulated by affecting the relative activities of various forms of α-galactosidase. The results of the present study together with those reported by other workers (43, 44, 82) on the *in vitro* association of low molecular weight forms of α-galactosidase to form the high molecular weight form enzyme in legume seeds

support the view of *in vivo* conversion by association/dissociation of low/high molecular weight forms of the enzyme that could occur during various physiological states of the seed. For example, extracts of green and immature *Vicia faba* seeds showed only a low level of monomeric forms of α-galactosidases. The level of activity increased during early seed maturation accompanied by a marked gain in specific activity (52,159). The later increase was related to the appearance of tetrameric form of the enzyme which normally displays a several fold higher specific activity. Further, these workers observed that extracts of seeds in the course of maturation show a gradual increase in tetrameric form with a concurrent decrease in monomeric form (159).

Although, *in vitro* and *in vivo* studies strongly suggest the phenomenon of interconversion of different molecular species of α-galactosidase in germinating and maturing chickpea seeds, the possibility of *de novo* synthesis of different molecular species of α-galactosidase can not be rule out. In this regard the results of cycloheximide, an inhibitor of protein synthesis (20), treatment were interesting (Fig 24, 26). During 24 h imbibition of seeds in water or 12 h after cycloheximide treatment the level of α-galactosidase activity in both untreated and cycloheximide-treated chickpea seeds was same and remained unchanged indicating a period of no or little

synthesis of the enzyme. Following this period while in the untreated seeds the α-galactosidase level increased continuously during the 144 h germination period, the enzyme level in the cycloheximide treated seeds first declined during the next 24 h of germination period and then started to increase. Thus, a period of 24 - 36 h after the cycloheximide treatment appeared suitable to study the in vivo conversion of different molecular forms of α-galactosidase in chickpeas.

The cycloheximide treatment seems to introduce two phases of development of molecular forms of α-galactosidase. In the early phase of germination period (30 h after cycloheximide treatment) the existing high molecular weight form (α-galactosidase I) appears to dissociate into low molecular weight enzyme (α-galactosidase II, Fig 26) which is identical to the pattern of enzymes in germinating cotyledons without cycloheximide treatment while in the second phase (78-126 h germination period after cycloheximide treatment) formation of high molecular form increases at the cost of low molecular form α-galactosidase. These results are in contrast to the pattern of α-galactosidase molecular forms of untreated seeds during germination but, in fact, resemble the isoenzymic pattern of maturing seeds.

The number of isoenzymes in a seed may be attributed to a number of things. This may be due to genetically

independent proteins, genetic variants (allelic), or heteropolymers of polypeptide chains that are bound non-covalently. Conjugation of other molecules with proteins or multimers of a single subunit may also be the causes. Results given in Table XVI show a remarkable variation both in the number and the relative concentrations of various species of α-galactosidase among genetically different varieties of chickpea. For example, whereas most varieties contained one high and two low molecular weight forms, a few of them like black variety chickpea $C_{235(4)}$ and $H_{208(2)}$ and local variety number 7 contained only the high molecular weight form of α-galactosidase. On the other hand all white varieties chickpeas tested lacked high molecular weight form of α-galactosidase, but contained a significant level of a novel very low molecular weight form (Mr = 25,000 daltons) of the enzyme. The very low molecular weight form α-galactosidase was also found to be present in some black varieties chickpeas, namely $C_{235(2)}$, $C_{235(5)}$, $H_{208(1)}$, $H_{208(5)}$, and local variety 1,2, and 5. The presence of very low molecular weight form α-galactosidase has also been reported in the following seeds: Ceratonia siliqua, Cyamopsis tetragonolobus, Glycine max, Lupinus angustifolius and Medicago sativa (79,119). Interestingly enough black variety $C_{235(5)}$ did not contain the normally existing low molecular weight

α-galactosidase IIa and IIb. It is suggested that the pattern of α-galactosidase molecular forms in dormant seeds of different varieties of chickpea and also in other legume seeds may form the basis of their biochemical characterization and classification.

## 6.0 SUMMARY AND CONCLUSIONS

The multimolecular forms of α-D-galactoside galactohydrolases (E.C. 3.2.1.22) of chickpea (Cicer arietinum) cotyledons have been investigated with a view of (i) isolation, purification and characterization of different molecular forms of α-galactosidase from germinating cotyledons of chickpea seeds, (ii) to understand the physiological function and developmental pattern of α-galactosidase isoenzymes, (iii) to study the interrelationship between various molecular forms of α-galactosidase in vitro and (iv) to compare the isoenzymic patterns of α-galactosidase in different varieties of chickpea seeds.

Chickpea cotyledons were found to contain 3 molecular forms of α-galactosidase, the high molecular weight form (α-galactoridase I) and two low molecular weight forms (α-galactosidase IIa and IIb). From the 6 days old germinating cotyledons the low molecular weight forms were purified to homogeneity by polyacrylamide gel electrophoresis using (i) acetone treatment (ii) pH precipitation, (iii) ammonium sulphate fractionation (iv) Sephadex gel filtration and (v) ion exchange chromatography on C M - cellulose.

The Sephadex gel filtration produced 2 peaks of α-galactosidase activity representing the high molecular weight and the low molecular weight enzyme species. The α-galactosidase II was further resolved into 2 distinct

species by ion exchange chromatography on C M-cellulose. These were designated as α-galactosidase IIa and α-galactosidase IIb according to their elution order. The molecular weight of α-galactosidase I, α-galactosidase IIa and α-galactosidase IIb, as determined by Sephadex gel filtration method, were 149, 600 daltons, 56, 000 daltonsand 50, 000 daltons, respectively. With SDS polyacrylamide gel electrophoresis under completely dissociated conditions α-galactosidase IIa yielded three protein bands corresponding to Mr=56,000 , 22,000 and 16,000 daltons. The α-galactosidase IIb gave two bands of molecular weights 40,000 and 18,000 daltons. The results indicated the subunit structure for α-galactosidase isoenzymes.

The properties characterising the high molecular weight form and low molecular weight form of α-galactosidase were determined by using p-nitrophenyl α-D-galactopyranoside as substrate. The pH optima of α-galactosidase I, IIa and IIb were pH 5.2, 5-5.2 and 5.5. The value of temperature co-efficient, $Q_{10}$ , between 25°C and 40°C for α-galactosidase IIa and IIb were 0.85 and 0.72 respectively.

The effect of temperature on the kinetic parameters of α-galactosidase IIa and α-galactosidase IIb are reported in terms of energy of activation (Ea), enthalpy

change ($\Delta H$), free energy change ($\Delta G$) and entropy change ($\Delta S$). Ea of α-galactosidase IIa and α-galactosidase IIb were 14.64 and 16.64 Kcal/mol, respectively. $\Delta H$ values were positive indicating that in all cases the reaction was endothermic. $\Delta G$ of α-galactosidase IIa and IIb were 1.17 and 0.77 Kcal/mol. $\Delta S$ values of α-galactosidase IIa and IIb were found to be 0.048 and 0.055 Kcal/mol/Kelvin respectively. The α-galactosidase IIa and α-galactosidase IIb were found to be glycoproteins with a carbohydrate percentage of 9.4 and 29.5, respectively, composed of N-acetyl glucosamine and mannose.

α-Galactosidase isoenzymes were immobilized in calcium alginate beads. The properties of immobilized enzymes were studied and compared with that of free enzyme. It was found that there was no shift in optimum pH of immobilized enzymes but they were having higher pH tolerence and greater thermal stability as compared to the free enzyme. The immobilized enzymes were having higher $K_m$ and lower $V_{max}$ values than the free enzyme. For example, the $K_m$ values of high molecular weight form, α-galactosidase I, and low molecular weight forms, α-galactosidases IIa and IIb, in the free form were 0.36 mM, 0.14 mM and 0.28 mM. Whereas in the case of immobilized enzymes these were 1 mM, 0.28 mM and 0.36 mM respectively. Similarily the $V_{max}$ of free α-galactosidase I, IIa and

IIb were 5.3 μmol/min/mg, 2 μmol/min/mg and 1.0 μmol/min/mg while in immobilized state these were 3.3 μmol/min/mg, 1.1 μmol/min/mg and 0.9 μmol/min/mg respectively. It was found that the enzyme was more stable in immobilized form when stored at 4°C at optimum pH.

The low molecular weight forms, α-galactosidase IIa and α-galactosidase IIb, when stored together as mixture associated to form a high molecular weight enzyme with nearly identical properties of α-galactosidase I. It was also found to be a glycoprotein having 25% carbohydrate content. On the other hand, when purified α-galactosidase IIa or α-galactosidase IIb were stored separately under similar conditions no transformation occurred indicating that the high molecular form was reconstituted as a result of association of α-galactosidase IIa and α-galactosidase IIb.

The relative concentrations of α-galactosidase I and α-galactosidase II vary with physiological state of the seed. The developmental patterns of α-galactosidases during germination and maturation were investigated. An examination of the Sephadex patterns and total enzymatic activity of α-galactosidases showed that α-galactosidase starts its formation at very early stage of seed development. In green immature seeds, the total α-galactosidase activity was very low and only the low molecular weight form enzyme was dominated. The activity of low molecular weight form increased with seed development. After 17 days of flowering a marked increase in total

activity of α-galactosidase occured and α-galactosidase I made its appearance. As the development proceeded 25th to 40 days after flowering the level of high molecular wieght form enzyme increased and the level of α-galactosidase II started to decline. In the later stages of seed maturations the high molecular weight enzyme became dominant with a very little amount of low molecular weight enzyme. In germinating seeds the pattern was completely reversed i.e. during germination the level of high molecular weight enzyme decreased with a corresponding increase in low molecular weight form. After the cycloheximide treatment, the pattern of α-galactosidase enzyme in germinating cotyledons was more like that of during the maturation process.

The α-galactosidase isoenzymic patterns of different varieties of seeds of Cicer-arietinum were compared in order to find a biochemical basis of characterization. It may found that the high molecular weight form of α-galactosidase was not present in all white varieties of chickpea seeds tested. In addition, the white variety contained a novel very low molecular weight form enzyme of Mr=25,000 daltons, which was not present in any of the black variety of seeds. All the black variety chickpeas contain high molecular

weight form α-galactosidase. These results indicated that the number of molecular forms and their relative amounts differ from species to species.

It is suggested that α-galactosidase isoenzymic patterns of chickpeas may form the basis of identification of genetically different species.

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