A STUDY OF PHYTASE SYSTEM IN CUCURBITA MAXIMA (PUMPKIN) COTYLEDONS DURING GERMINATION

A THESIS

submitted in fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

By

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July, 1985

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "A STUDY OF PHYTASE SYSTEM IN CUCURBITA MAXIMA (PUMPKIN) COTYLEDONS DURING GERMINATION" in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy, submitted in the Centre of Biosciences, University of Roorkee, Roorkee, is an authentic record of my own work carried out during the period from September 1981 to July 1985 under the supervision of Dr. C.B.Sharma, Professor and Head, Centre of Biosciences, University of Roorkee, Roorkee and Dr. S.P.Srivastava, Professor and outgoing Head, Chemistry Department, University of Roorkee, Roorkee.

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

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ABSTRACT

The present study concerns with the isolation, characterization, development and mode of action of multiple phytase species from Cucurbita maxima germinating cotyledons.

Six molecular forms of phytase were separated and purified to homogeneity by SDS polyacrylamide gel electrophoresis (SDS PAGE) from the germinating Cucurbita maxima cotyledons using acetone and ammonium sulphate fractionation, Sephadex gel (G-150) filtration and ion exchange chromatography on DEAE- and CM-cellulose. Gel filtration produced two peaks of phytase activity representing the high molecular weight (phytase I) and the low molecular weight (phytase II) species. The phytase I was further resolved into 5 distinct species on CM-cellulose. These were designated as phytase IA, IB, IC, ID and IE according to their elution order. The phytase II emerged as a single peak both from DEAE and CM-cellulose columns. The molecular weights of phytase IA, IB, IC, ID, IE and II as determined by SDS PAGE were found to be 60,256; 63,096; 43,657; 77,625; 38,905 and 29,512 Daltons respectively.

Some other properties characterising the multiple forms of phytase (IA, IB, IC, ID, IE and II) were determined using phytic acid as substrate. The pH optima curves for all the enzymes were sharply peaked at 5.0, 4.8, 5.0, 5.2, 4.4 and 5.6. The temperature optima of phytase I isoenzymes (IA, IB, IC, ID and IE) lie between 45-50°C but for phytase II it was

found to be sharp at 55°C. The values of temperature coefficient Q_{10}, between 30° and 40°C for phytase isoenzymes were found to be ranging from 1.3 to 2.3. All the phytase I isoenzymes were thermally stable upto 40°C, but phytase II was stable upto 50°C.

The effect of temperature on the kinetic parameters of different phytase species are reported in terms of energy of activation (E_a), enthalpy change (ΔH), free energy change(ΔG) and entropy change (ΔS). Both K_m and V_{max} values increase with increasing temperature. E_a of phytase isoenzymes are different ranging from 3685 to 6909 Kcal/mole. ΔH values are positive indicating that in all cases the reaction is endothermic. ΔG values for phytase isoenzymes were found to be varying from 923 to 1845 Kcal/mole. ΔS values were found to be very low suggesting that phytases undergo little conformational change during enzyme substrate complex formation. The Arrhenius plots of log V_{max} versus 1/T in the temperature range 30°-50°C were found linear indicating that E_a remains constant during reactions.

The substrate affinity of different phytase species were determined towards different myoinositol polyphosphates on the basis of V_{max}/K_m values. The data indicated that the myoinositol hexaphosphate is the most suitable substrate for enzyme II followed by IE and IB. Similarly myoinositol pentaphosphate is preferably hydrolysed by enzyme IE followed by enzyme II. The myoinositol tetraphosphate is a preferred substrate for both enzyme II and ID but the myoinositol

triphosphate is the most suitable substrate for enzyme II. Diphosphates and monophosphates show preference for enzyme IE amd II. In addition, the phytase II exhibits high substrate affinity for all inositol polyphosphates which may be of physiological significance, as this enzyme makes its appearance at a very late stage of germination when both the level of phytase I activity and the concentration of phytase are very low.

The mode of dephosphorylation of myoinositol hexaphosphate (MIP_6) by individual phytase isoenzyme was studied *in vitro*. The phosphorus-containing intermediates formed during hydrolysis of MIP_6 were isolated by ion-exchange and paper chromatography and identified by periodate oxidation and acid catalysed phosphate migration across cis OH groups and by IR spectroscopy of sugar alcohols formed on periodate cleavage of myoinositol phosphates followed by reduction and dephosphorylation with $NaBH_4$ and HCl, respectively. These results showed that the dephosphorylation of phytic acid by all the molecular species of phytase, proceeds in a stepwise manner.

On the basis of kinetic data and the pattern of development of different phytase isoenzymes and assuming that the relative concentrations of different phytases together with substrate affinity, nature and relative substrate concentrations should determine the course of phytate dephosphorylation *in vivo*, the possible role of each phytase isoenzyme has been suggested (Fig. 1). For example, phytase IB seems to be

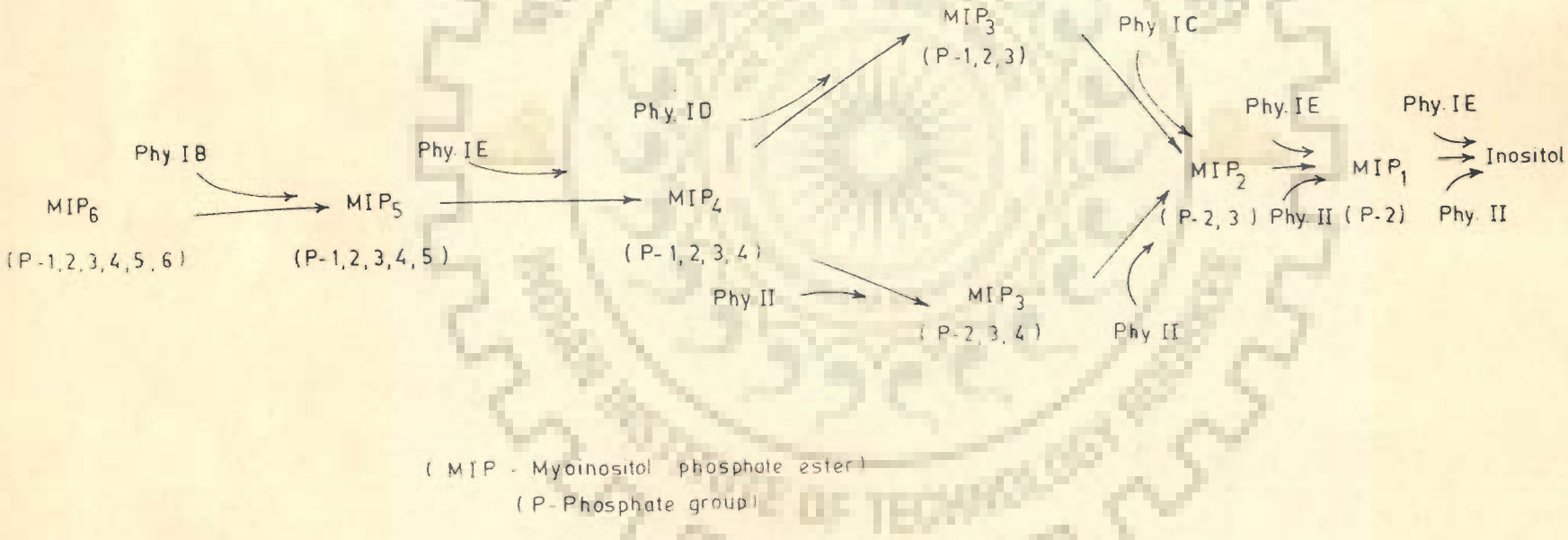

Fig. 1 - Dephosphorylation Pathway of Phytic Acid

involved in the selective dephosphorylation of MIP_6 at position No. 6 to yield corresponding MIP_5 and the lower molecular forms of phytase being more specific for the lower inositol phosphates.

The developmental pattern of the multimolecular species in germinating pumpkin cotyledons was investigated by separating the diffcrent molecular forms and analysing for their relative levels of activities at different stages of germination. No phytase activity was present in the unimbibed intact cotyledons but the early phase of germination period, between first and seventh day, was marked by the high rate of synthesis of the high molecular weight enzyme, phytase I, species with very little synthesis of low molecular weight enzyme, phytase II. In the later stages of germination, between 9th and 15th day, there was a sharp decline in the level of phytase I activity with simultaneous rise in phytase II activity level. It is remarkable to note that in the very onset of germination (first 12 hours of germination), the isoenzyme IB was found to account for more than half of the total phytase I activity followed by phytase IA (25 %). However, the activity level of IB enzyme declined rapidly and became nearly insignificant in about 48 hrs of germination. These results indicate that in the very early period of germination, isoenzyme IB is responsible for dephosphorylation of myoinositol hexaphosphate, the primary form of reserve phosphate.

The enzyme IA shows somewhat a different pattern of

development. Its activity level increases upto 48 hrs contributing about 50 %. of the total phytase I activity. This state is maintained upto 180 hrs. After this peak period the level of its activity starts declining slowly indicating that the enzyme IA plays a major role in the phytate metabolism during 48 to 180 hours germination period when the activity of all other phytase I isoenzymes was low. At this moment it is not certain if the enzyme IA and IB are structurally related. The molecular weight data and developmental pattern, however, indicate such relationship, i.e. IB may be the precursor of IA.

Enzyme IC and ID showed minor activities. Their activities were found to be maximum at 84 and 48 hours of germination respectively. After that they started declining and became insignificant by the end of germination (15 days).

The enzyme IE was found to follow yet another developmental pattern as its activity increases progressively from merely 10 %. after 12 hrs, upto 70 %. of phytase I activity at the end of 372 hours of the germination. So it seems that enzyme IE plays rather a supplementary role for the enzyme IA especially in the later stages of germination.

The synthesis of phytase II enzyme (low molecular weight) starts only after 36 hours of germination and unlike phytase I its activity goes on increasing through the total germination period of 15 days. In fact, the activity level of phytase II clearly becomes dominant between 12th and 15th day of germination. Like phytase IE, the role of this enzyme also

seems to supplement the phytase IA in the later period of germination when only lower inositol phosphates such as tri-, di- and monophosphates would be available in relatively higher concentrations than phytate as substrate.

Thus, it seems that the relative concentrations of different isoenzymes are related to the stage of germination and may be responsible for the regulation of the overall phytate metabolism in germinating cotyledons of pumpkin seeds.

ACKNOWLEDGEMENTS

The author avails this opportunity to express her deep sense of gratitude to Dr. C.B.Sharma, Professor and Head, Centre of Biosciences and Biotechnology and Dr. S.P.Srivastava, Professor and formerly Head, Chemistry Department, University of Roorkee, Roorkee for their expert guidance, valuable criticism, helpful suggestions and constant encouragement throughout the completion of this work.

Author feels indebted to Dr. C.B.Sharma who inspite of being very busy took pains to arrange research facilities even from abroad without which this work could not have been completed. Author is also grateful to Dr. S.P.Srivastava who has been good enough to arrange CSIR sponsorship and spare research facilities in the Chemistry Department for this work.

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CHAPTER - I

INTRODUCTION

The phytase (myoinositol hexaphosphate phosphohydrolase EC 3.1.3.8) catalyzes the hydrolysis of phytate (myoinositol hexaphosphate), the main form of the reserve phosphate in seeds and grains (65), into inorganic phosphate (P_i) and myoinositol (148-150). The enzyme occurs widely including microorganisms (16-19,23). The germinating seeds are particularly rich source of phytase where it is largely found in the endosperm. But in wheat grains it is also found in the scutellum and germs (63). In dormant seeds it is completely absent and the de novo synthesis of the enzyme starts only after several hours (12-24 hours) of imbibition of seeds and initiation of germination process. The information on the mode of the development of phytase enzyme in the germinating seedlings is, however, still quite vague. A correlation between the degradation rate of phytate, level of P_i and level of the phytase activity has been observed in germinating seeds indicating that the de novo synthesis of phytase and the level of its activity may play a key role in the mobilisation of phosphate reserves from the storage tissues (cotyledons and endosperm) to meet the phosphorus requirement of the growing plant during the early period of germination and plant development (31,34,37).

Several workers have investigated the dephosphorylation of phytate by phytase in vitro (85,100,102,104,105).

The elegant work of Tomlinson and Ballou and later by some other workers (72), clearly demonstrated that the complete dephosphorylation of phytate into P_i and myoinositol by wheat bran phytase occurs in a stepwise manner involving a multisubstrate pathway (74,75,92,93,95). Since the phytase preparations used by these workers were not pure, it was not possible to assess the correctness of the multisubstrate pathway. Nevertheless such a pathway suggests the participation of more than one enzyme. That, this may be so, is supported by the following data: firstly, the phytate molecule contains six different myoinositol monophosphoester bonds on which phytase enzyme can act, secondly, the presence of multiple forms phytase has been demonstrated in several seeds and grains (74-76); and thirdly, the structures of the lower myoinositol polyphosphates produced during dephosphorylation of phytate by phytases derived from a variety of sources were found to be different (26).

In order to determine the role of multiple phytases in the dephosphorylation of phytate molecule it would be necessary to isolate and purify various molecular forms of phytase from a suitable biological source and the phytate degradation pathway for each form of the enzyme to be investigated. In addition, studies on the developmental pattern and the substrate affinities of each phytase species especially towards lower myoinositol polyphosphates will also be useful in assigning the specific role of the individual enzyme species in the mobilisation of phytate phosphorus in germinating seeds.

Recently, Goel and Sharma (151) reported the presence of multiple forms of phytase in the germinating cotyledons of Cucurbita maxima seeds. But the mode of dephosphorylation, development and substrate specificity of the different species were not investigated in sufficient detail. Further, the total phytase activity was not accounted by relative abundance of various molecular forms. Hence, the role of the multiple phytase species is not clearly understood. In view of the above the present study was undertaken to investigate the phytase system in the Cucurbita maxima seeds with following objectives:

1. To prepare sufficient quantity of the different molecular species of phytase in homogeneous form from the Cucurbita maxima cotyledons of suitable germination stage.

2. To isolate, purify, identify and determine the structure of various myoinositol polyphosphates formed as intermediates during the in vitro dephosphorylation of phytate by the different species of phytase.

3. To study the kinetic parameters and the relative substrate affinities of various phytase forms towards different myoinositol polyphosphate intermediates as compared to phytate.

4. To establish the major pathway for the phytate degradation in Cucurbita maxima seeds.

5. To study the mode of development and role of the individual phytase species in the mobilisation of phytate-phosphorus during a defined germination period when the developing plant depends entirely on the endogeneous supply of nutrients including P_i.

CHAPTER - II

LITERATURE SURVEY

2.1 INTRODUCTION

Phytase (myoinositol hexaphosphate phosphohydrolase, E.C. 3.1.3.8) plays a vital role in mobilising the phytate phosphorus, the primary reserve organic phosphate in seeds, during seed germination (148-150). In the course of germination and sprouting of seeds, the reserve phosphate is enzymatically degraded to inorganic phosphate and myoinositol for utilization by the growing plant. Several excellent comprehensive reviews on phytases have been published in recent years (11,12,74,151). In this chapter the current status of the chemistry and biochemistry of both phytate, the naturally occurring substrate, and the control mechanism of phytase action have been reviewed.

2.2 OCCURRENCE

2.2.1 Phytase

Phytases widely occur in nature including microorganisms (57-60) and some animals (61,62) but are particularly rich in germinating seeds (1 to 4, 73,87,88,90,91). In general, ungerminated seeds contain little or no phytase activity but during the early period of seed germination, the enzyme activity increases sharply (40,41,64). Phytase activity is found to be maximum in cotyledons and endosperm (7,6) but in wheat grains it is also found in scutellum and germs(63

Phytase enzymes from various beans, rice, wheat, barley have been isolated and characterised (5 to 7,9 to 15). Recently phytases from Aspergillus ficuum NRRL 3135 (16,17), Aerobacter bacillus, Aspergillus flavus, A. Carbonum (18), pseudomonas (19), Aerobacter aerogenes (23) have been studied. The enzyme has also been found to exist in animal systems such as mucosa of small intestine of rat (21,22) and rumen (20). The phytase activity was also observed in fermentation processes (24,25).

2.2.2 Substrate

Myoinositol hexaphosphate (MHP), the natural substrate for the phytase, widely occurs in nature in form of its Ca, Mg, Fe complex salts called as phytin (67). No case has been reported of a seed in which phytin is completely absent (65). Ripening seeds are rich source of phytic acid constituting 75 to 80 % of organic phosphate (2,3,5,29,109-113). In ripening seeds phytic acid occurs in cotyledons (114) localized mainly in aleurone grains (7,35,36,115-117,66). It is also found in embryo and scutellum (115). Phytic acid is also a part of animal and human dietary (20,22,48). Phytic acid's presence in various types of soil is quite well known for sometime (18,40,118).

2.2.3 Biosynthesis of Phytic Acid

During ripening of seeds, total phosphate and phytin phosphate increase rapidly while inorganic phosphate decreases

and thus myoinositol hexaphosphate (MHP) reaches maximum in fully mature seeds (2,3,112,113). The main component of organic phosphate is myoinositol hexaphosphate in ripe seeds. Accumulation of MHP in seeds occurs by multistep biosynthesis of MHP (113). Phytic acid was formed in aleurone layers of rice seeds by incorporating orthophosphate ^{32}P and myoinositol-^{14}C. However, when ^{14}C-glucose was incubated, no incorporation of ^{14}C in phytic acid was observed indicating that phytic acid is formed by phosphorylation of myoinositol (116). As will be mentioned below when ^{14}C-glucose-6-phosphate was used, the radioactivity was found in phytate.

The biosynthesis of phytic acid is known to be catalysed by enzymes causing a stepwise phosphorylation of myoinositol or IL-myoinositol-1-phosphate into phytic acid with ATP as phosphate donor and in the presence of some Mg^{2+} and Mn^{2+} ions and isoenzymes (110,117).

By the action of glucose-6-phosphate cyclase on glucose-6-phosphate-6-^{3}H is cyclized into D-inositol-1-phosphate involving the activation and release of H,bound to C-6(119,120) The action requires presence of NAD^{+} and NH_4^{+}. In the presence of Mg^{2+} ions myoinositol-1-phosphate is further hydrolyzed to myoinositol (121,120).

When the rate of phytin synthesis was determined during prechlorophyll, chlorophyll and post chlorophyll stages of maturation period of wheat (122), phytate concentration was found to be double during chlorophyll stage. Further it

became 75 % of total phosphorus during post chlorophyll stage. The actual process of the bisynthesis of phytate is, however, unknown. A clear understanding of the phytic acid biosynthesis will be very useful for the understanding of the regulatory mechanism and physiological role of phytic acid.

2.3 STRUCTURE OF THE NATURAL SUBSTRATE

The natural substrate for the enzyme is myoinositol hexaphosphate, phytate (MHP). Two different structures were proposed for this molecule. One with 18 atoms of acid hydrogen $C_6H_{24}O_{27}P_6$ proposed by Neuberg (79) and second with 12 atoms of acid hydrogen $C_6H_{18}O_{24}P_6$ suggested by Anderson (78) as shown in Fig. 2 . The latter structure was found to be correct which was confirmed by Johnson and Tate by ^{31}P nuclear magnetic resonance spectroscopy (77).

Inositol hexaphosphate exists in aqueous solutions in either two conformations depending on pH (107). The low pH form possesses one axial and five equatorial phosphates whereas high pH has inverted 5 axial and 1 equatorial structure. Equal amounts of two conformers coexist at pH 9.4±0.1. The ^{31}P NMR spectra as a function of pH reveals that 3 least acidic phosphate protons have apparent pKa values of 9.2 to 9.6. The inositol hexaphosphate ring inversion is most probably triggered by one or more of these acid dissociation steps. Raman spectra are sensitive both to acid dissociation and conformation. The Raman spectra of solid Ca_6MHP is

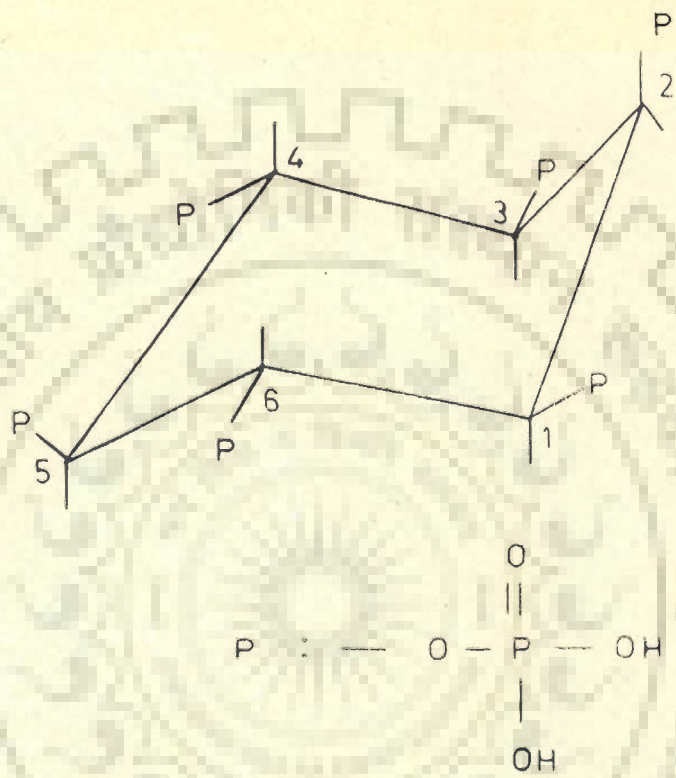


Fig. 2 - Structure of Phytic Acid

characteristic of the one axial/5 equatorial conformer in contrast to 5 axial/1 equatorial structure of dodeca-sodium salt.

Phytic acid contains 31 or 35 molecules of water of cystallization (77). Its water of crystallization reduces to 3 molecules by heating at 100°C and 1 molecule by heating at 145°C but complete dehydration does not occur (169,170). Further, heating of the compound above 150°C causes decomposition.

2.4 ROLE OF PHYTIC ACID IN DIFFERENT METABOLIC SYSTEMS

Phytic acid plays an important role in controlling mineral balance in human nutrition. Phytic acid when present in food, inhibits, absorption of Fe, Zn, Ca, Na, Cu (43,47, 48 to 50) by human intestine.

The chelating effect of phytic acid controls the effect of metal ions in various biological systems. Pretreatment of human tooth enamel with phytate solution inhibited subsequent mineralization (51). Thus phytate when applied as mouth washes decreased dental plaque formation (52,53). It is also incorporated into dental cements (54,55) and compressed apatite disks used as models for tooth enamel (46). Inositol phosphates also prevent tissue mineralization, thus they are pathogenetically important in curing certain disorders of mineral metabolism (56). A cream comprising of 6 ml 0.3 % aqueous guar gum, 5 g basic bismuth phytate, bisubnitrate can be used in treatment of

gastrointestinal ulcers, gastritis and diarrhoea(57-58). Also it has been used in curing acne, improving skin color, blood circulation, fingernail growth (61) and diabetes (59,60).

Partial hydrolyzates of MHP are also potent inhibitors of calcification in vitro of ricketic rat cartilage and aorta (125). A synergistic combination of phytic acid and polyacrylic acid makes a membrane which is used up in reducing Ca, Mg, or Fe in water purification by reverse osmosis (126). Sodium phytate reduces the effect and lowers the blood concentration of drugs which they do not precipitate (139). Phytic acid has also been useful in preventing decoloration and in preservation of food and vegetables, noodles, soy sauce, fish meal, alcoholic drinks, fruit juices, edible oils, etc., (127 to 129, 141,142), and in obtaining foam and scum free coffee (143). It is also used in cosmetics for skin care (130).

Phytic acid combines with proteins to form insoluble protein phytates in acidic medium, thus it inhibits pepsin proteolysis (44,131) and phytic acid hydrolysis (24). It also accounts for the turbidity of rape seed oil (133) and precipitation of serum albumin (140). It also inhibits α-amylase activity (132). MHP interacts with haemoglobin (144 to 147) forming 1:1 complex. Thus, it reduces the oxygen affinity of haemoglobin.

Addition of phytate to glass cement shortens setting time and increases compressive strength (134). It is also advantageous in galvanizing steel strips (135 to 137) as it

improves their corrosion resistance and adhesion property. Baths containing metal ions and phytate can be used in deposition of metals (138).

2.5 MODE OF DEPHOSPHORYLATION OF PHYTATE

Although several mechanisms for the phytase action on the phytic acid molecule have been proposed, most evidence favours the multichain mechanism in which the enzyme acts randomly on all the positions of phosphate groups in substrate molecule.

Enzymic hydrolysis of phytic acid in germinating seeds occurs stepwise, by successive liberation of phosphoric acid and through intermediate formation of penta-, tetra-, tri-, di- and monophosphates of myoinositol, the final products being myoinositol and phsphoric acid (23,26,29,31,32,35,37, 68,69,73). The released free orthophosphate is used up in oxidative and photosynthetic phosphorylation (31) and in synthesis of phospholipids (34). There is however, some doubt about the direct transfer of phosphate group from MHP to ribonucleoside di-phosphates. These observations could not be reproduced.

Also the decrease in phytin level during germination is accompanied by an increase in levels of such compounds as hexose phosphates, ADP, ATP (37). In peanut seeds, phytic acid is localized in aleurone grains and acts as a binding agent of Mg (66). Thus phytin serves as an important reserve of metal ions too for germinating seeds (65,123,124).

However, the mechanism of dephosphorylation of inositol hexaphosphate has not been extensively examined. Also this mechanism is found to proceed by different pathways in different biological systems (26).

A general degradative scheme for the action of wheat phytase was given by Tomlinson and Ballou (68). A twenty fold purified wheat phytase was used. The myoinositol phosphate intermediates were separated by ion-exchange column chromatography on Dowex-1 (Cl^-), using a linear gradient, into mono-, di-, tri- and tetra- inositol phosphates. The intermediates in reaction identified were myoinositol-1-and 2-monophosphate; myoinositol-1,2 diphosphate; myoinositol-1,2,3 and -1,2,6 triphosphate and myoinositol-1,2,5,6 tetraphosphate and another tetraphosphate which could not be recognized. The structure of pentaphosphate could not be determined as it was eluted out along with hexaphosphate. But it was not possible to assess the validity of multiple substrate pathway scheme as the phytase preparation was not pure. Nagai and Funahashi (62) using a 1500 fold purified wheat bran phytase preparation confirmed the results. The action of phytase derived from a variety of sources were compared by Uehara, et al (69) and large differences in the relative amounts of myoinositol polyphosphates produced were found after the completion of 50 %. reaction. In addition, the structures of myoinositol penta-, tetra-, tri- and diphosphates produced by different phytases were found to be different (74,75,92,93,95). Thus, it seems that dephosphorylation of phytic acid by different phytases proceeds

via different pathways. The multiple substrate pathway indicates the presence of multiple forms of phytase. Recently, it has been demonstrated that multiple forms of phytase are present in wheat (74,75) and rice brans (76). Their structural relationship and physiological significance is not known.

The actions of phytases from different sources as potatoes (74), rice hulls (74), onions (74), fungus A.ficcum NRRL 3135 (11), pseudomonas (19), Aerobacter aerogenes (23), have been studied.

Some soil microorganisms have also been isolated which hydrolyse soil phytate to myo-inositol and P_i.d-l inositol and scyllo-inositol also do occur alongwith them in soil (18,40). But the mechanism of hydrolysis has not been fully investigated.

2.6 REGULATION OF PHYTASE ACTIVITY

The exact mechanism of the regulation of phytase activity in biological tissues is virtually unknown. It has been reported that phytase activity is repressed both in vivo (89,102) and in vitro (103) by orthophosphate. The increase in phytase activity in wheat embryo was sensitive to puromycin (86). On the basis of these results Sartirana and Bianchetti (86) proposed that in the first stage of seed germination the very low level of inorganic phosphate would favour the synthesis of the enzyme. Then, as a result of increased

rate of phytin breakdown, the level of P_i would progressively increase leading to the repression of phytase synthesis.

The available data of the control of phytase activity in germinating seeds and utilization of phytin phosphorus (85,100,102,104,105) suggests that the de novo synthesis of phytase occurs during the germination and the optimum period appears to be between 48 and 72 hours after the seeds were planted for germination under normal conditions (86,100).

2.7 PURIFICATION AND PROPERTIES OF PHYTASE

Although this has been known for long, phytase occurs widely and plays an important role in metabolism of seed. In most studies including the course of the enzyme action only the partially purified preparations have been used (10,29). In fact, as will be shown below, it is only observed in a very few studies that the systematic purification of the enzyme has been attempted. The most highly purified preparation of a plant phytase to-date was achieved by Nagai and Funahashi (62). They obtained a 1500 fold purification of enzyme from wheat bran by $(NH_4)_2SO_4$ fractionation, organic solvent precipitation, and ion exchange column chromatography. But the enzyme has very high activity towards other substrates as well such as ATP, ADP, pyrophosphate, fructose-1, 6-diphosphate, phenyl phosphate and others, in addition to its activity towards phytic acid. The homogeneity of the enzyme was not clearly established either.

Later Powar and Jagannathan (64) obtained a purified phytase from Bacillus subtilis by gel filtration, acetone and methanol fractionation and adsorption on Ca-phosphate gel. This bacterial phytase enzyme was highly specific as it showed no activity with α-glycerophosphate, β-glycerophosphate, diphenyl phosphate, glucose-1-phosphate, fructose-1, 6-diphosphate, pyrophosphate, NADP, ATP, ADP or AMP. It was however, not determined, whether this enzyme could hydrolyse phytic acid completely into inositol and P_i. In other words the degradation pathway of phytate molecule was not investigated. Recently, the affinity gel prepared by coupling phytic acid and aminohexyl sepharose, chromatography was also used to purify phytase (27).

In the course of purification by gel filtration (16) or by ion-exchange chromatography (26), existence of the multimolecular species was also observed. In most cases, however, number of the enzyme forms was limited to two (3, 27,39).

Despite the clear demonstration of the existence of multiple forms of phytase in plant seeds, the isolation and purification of different forms of phytase has not been achieved except for one report by Goel et al (151). This study is also incomplete in many respects. For instance, the reaction pathway for each form of enzyme was not investigated nor was there any attempt to determine the substrate affinity. As a result, the role of multiple forms of phytase remained obscurred.

Crude and partially purified phytase enzymes from various sources have been characterized (10-12,15,16,26, 28-30). Their optimum pH is found to be in acidic range. K_m values are specific for phytases of different sources, Fe^{3+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ag^{+}, Ni^{2+}, CO^{2+}, inorganic phosphate, F^{-} ions inhibit the enzyme activity. Oxalate citrate, EDTA and tartrate have no effect. Divalent ions are not required for activity. According to Peers (108) Zn^{2+} and Mn^{2+}, act by precipitating phytate ions. NaN_3, oxalates and $CaCl_2$ activate the enzyme activity (108). The activation by $CaCl_2$ may be due to removal of P_i from solution as a calcium phosphate precipitate.

Lower members of inositol phosphates are better substrates but inositol monophosphate is hydrolyzed at the slowest rate (28,10). Isolated enzyme is stable at $0^{o}C$ (10) and has a high optimum temperature $45-57^{o}C$ (10,12,15,30). Energy of activation of this enzyme is positive. Molecular weight of purified phytase as determined by SDS polyacrylamide gel electrophoresis (29) varies from source to source, between 35,000 and 70,000 daltons.

2.8 SUMMARY

The present literature survey on phytic acid and phytases has revealed the following points:

1. Phytate plays an important role in the metabolism of seeds. It is also of physiological importance with regard to the metal chelating action which has not been investigated in sufficient details.

2. The phytases from different origins differ widely in their mode of action towards phytic acid. The relationship between structure, function and mechanism of action is unknown.

3. The factors involved in the regulation of phytase activity both *in vivo* and *in vitro* have not been fully investigated.

4. Although, multiple phytase systems have been known to be present in germinating seeds, their physiological significance, mode of development and reaction pathway have not been investigated. Neither they have been isolated and purified and fully characterized.

CHAPTER - III

ISOLATION AND PURIFICATION OF THE MULTIMOLECULAR FORMS OF PHYTASE ENZYME IN GERMINATING COTYLEDONS OF CUCURBITA MAXIMA

3.1 INTRODUCTION

In germinating seeds the stored phosphorus is mobilised by the action of phytase (myoinositol hexaphosphate phosphohydrolase., EC 3.1.3.8) enzyme which hydrolyses the phytate into myoinositol and inorganic phosphate for use by the growing plant. Recently, multimolecular species of the phytase showing varied degree of specificity towards myoinositol hexaphosphate have been shown to exist in several germinating seeds (74,75,76,151). Further, these enzymes species have been implicated to be involved in the complete dephosphorylation of phytate to facilitate the mobilization of phytate-phosphorus during the early period of plant growth (23,26,29). This chapter concerns the isolation and purification of six distinct forms of phytase enzyme from germinating Cucurbita maxima seeds.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Sodium phytate ($C_6H_6O_{24}P_6Na_{12}$) was obtained from Sigma Chemicals, St. Louis, (U.S.A.). Acrylamid and N,N,N',N'-tetramethylathylendiamin were bought from Serva, Feinbiochemica Heidelberg, (Germany) and amidoblack from E.Merck

Darmstadt, (Germany). Sephadex G-150, bovine serum albumin (BSA) and CM-cellulose were purchased from Sigma chemicals (USA). DEAE cellulose was obtained from Bio-Rad Laboratories, Richmond, (California).

All other chemicals used in the present study were of analytical reagent grade. Pumpkin seeds, the test organisms, were obtained from the local seed store.

3.2.2 Methods

3.2.2.1 Enzyme Assay

Phytase activity was assayed by measuring the amount of inorganic phosphorus liberated from phytic acid substrate. The reaction mixture contained the following in the final volume of 2 ml : 0.50 mM of phytic acid, 50 mM sodium acetate buffer (pH 5.0) and 5-15 units of phytase enzyme (0.05 mg-0.20 mg protein). The enzyme action was started by adding the buffered substrate solution to the reaction mixture and then incubated at 40°C for 30-60 min. Enzyme activity was terminated by adding cold 20 % trichloroacetic acid. The denatured protein was removed by centrifugation and the amount of P_i was measured by the method of Fiske and Subba Row (152). The intensity of the blue colour developed was measured at 690 nm by a colorimeter. Controls both without substrate or without enzyme were incubated and processed concurrently with samples.

One unit of enzyme is the amount of enzyme in mg

which liberates one μ mole of inorganic phosphorus (P_i) per minute under assay conditions.

3.2.2.2 Protein Determination

Protein was determined by the method of Lowry et al. (153) using BSA (10-100 μg) as standard. The colour was developed with Folin and Ciocalteu reagent (1:2 dilution v/v) and the colour density was measured at 660 nm. The amount of protein in the enzyme samples was computed by referring to standard protein curve prepared concurrently.

3.2.2.3 Germination of Seeds

Seeds were germinated in dark at 35°C under asceptic conditions (102). Surface sterilized seeds were uniformly spread over four layers of cheese cloth in a tray and soaked with water and allowed to germinate in dark at 35°C. After germinating for the specified period of time (10-12 days), cotyledons were separated from the seedlings for enzyme extraction.

3.2.2.4 Enzyme Preparation

All the operations were carried out at 0-4°C unless otherwise stated. Germinating cotyledons were homogenized in ice-cold 50 mM acetate buffer of pH 5.0 (1:5 w/v) using Waring blender for about 1 min. The homogenate was centrifuged at 12,000 Xg for 30 min . The clear homogenate was dialyzed over night against the same buffer and subjected to 30 %. $(NH4)_2$ SO_4 precipitation. The protein fraction

precipitated was removed by centrifuging at 45,000 Xg for 50 min. Since this fraction was devoid of the phytase activity, it was discarded. To the supernatant sufficient amount of $(NH_4)_2 SO_4$ was added in order to achieve 80 % saturation. Protein precipitate was separated by centrifuging at 45,000 Xg for 60 min. It was dissolved in minimum volume (2-5 ml) of 50 mM sodium acetate buffer pH 5, dialyzed and the enzyme activity was measured. The total activity of phytase was found to be present in this fraction. It was used in prepurification and developmental studies of the total phytase activity.

3.2.2.5 Purification of Phytase Enzyme

3.2.2.5.1 Acetone Treatment

All operations were carried out at 0-4°C, unless otherwise indicated. 200g of cotyledons were homogenized in Waring blender with 1 litre of chilled acetone (-10°C) and filtered through Buchner funnel. The acetone powder was dried under vacuum and stored in desiccator at -20°C until used. 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 acetone powder could be stored in vacuum dessicator at -20°C for several months without any loss of phytase activity.

3.2.2.5.2 Preparation of Phytase Extract

Acetone powder (100g) was extracted with 500 ml of 50 mM sodium acetate buffer (pH 5.0) for 12 hour. The homogenate was filtered through four layers of cheese cloth and centrifuged for 40 min at 12,000 Xg. The clear supernatant was referred to as crude enzyme extract.

3.2.2.5.3 Ammonium Sulphate Fractionation

The clear supernatant obtained above was subjected to ammonium sulphate fractionation. To the enzyme extract sufficient amount of ammonium sulphate was added gradually with constant stirring to obtain 30 % saturation. It was kept overnight at 4°C and then centrifuged at 45,000 Xg for 60 min to remove the protein precipitate. The protein fraction was found to have no phytase activity. Further addition of ammonium sulphate to the supernatant was carried out, so as to bring 80 % saturation. It was kept for 12 hours and then centrifuged for 60 min. at 45,000 Xg. The protein residue obtained was dissolved in minimum volume (5-10 ml) of acetate buffer of pH 5.0 and dialyzed for 24 hours against same buffer and then tested for phytase activity. This was found to contain nearly all the phytase activity.

3.2.2.5.4 Gel Filtration on Sephadex G-150

Sephadex G-150 gel was suspended in 50 mM sodium acetate buffer of pH 5.0 and allowed to swell for 4-5 days. The floating fine particles were removed by repeated

decantation. Finally the Sephadex slurry was deaerated with the help of a suction pump. It was then packed under gravity in a double walled glass jacketed column (1x90 cm) fritted with glass wool at bottom to a column height of 70 cm. The column was fully equilibrated with the elution buffer (50 mM sodium acetate buffer, pH 5.0) for about 18 hours or until the pH of the buffer at the entry and the buffer discharged from the column, was same.

A 1.5 ml sample (Ca 500 mg protein) was applied to the previously equilibrated column and eluted with same buffer used for equilibration. The temperature of column was maintained at 4°C by circulating ice cold water in outer jacket of column. Two ml fractions were collected at a flow rate of 6 ml/hr. Aliquots (0.1 ml) from each fraction were analysed for protein and phytase activity. Two major peaks of enzyme activity were obtained. These were referred to as phytase I (high molecular weight) and phytase II (low molecular weight) as the high molecular weight fractions were eluted first (154,171).

These fractions with phytase activity were pooled separately, brought to 100 % saturation with $(NH_4)_2 SO_4$ and centrifuged for 60 min at 45,000 Xg. The precipitate was dissolved in 25 mM Tris-HCl buffer (pH 7.2) and dialyzed for 12 hour against same buffer.

3.2.2.5.5 DEAE Cellulose Column Chromatography

The DEAE cellulose (10g) ion exchange resin was washed

well with water and then 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 0.025 M Tris-HCl buffer (pH 7.2), and packed in a glass column (2x50 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 0.4 ml/min.

A 5 ml aliquot (Ca 100-200 mg protein) of the dialyzed fraction from Sephadex G-150 was applied to the column. The unabsorbed proteins were removed by washing the column with 200 ml of 0.025 M Tris-HCl buffer (pH 7.2). The absorbed proteins were then eluted by batchwise elution through 40 ml of each of the salt gradient ranging from 0.05 M to 0.50 M in same buffer. The flow rate was 0.4 ml/min and 2 ml fractions were collected. Aliquots (0.1 ml) from each fraction were analysed for protein and the enzyme activity. The fractions containing individual activity peaks were pooled and the protein was reprecipitated by $(NH_4)_2SO_4$ at 100 % saturation and centrifuged. Single peak was obtained in case of phytase I and phytase II. This fraction was dissolved in minimal volume of 25 mM acetate buffer, pH5, and dialyzed for 18 hour against the same buffer.

3.2.2.5.6 CM Cellulose Column Chromatography

Before filling the CM cellulose column, the cation exchanger was subjected to the following procedure in order to achieve optimum resolutions. 15 g material was suspended in 500 ml 0.25 M HCl and allowed to swell for 30 min with occassional slow stirring. It was then filtered on a Buchner funnel, rinsed with deionized water and resuspended in 500 ml 0.25 M NaOH and let it stand for 10 min. It was again filtered and thoroughly rinsed with deionized water. Washing with HCl and rinsing with deionized water was repeated. The material was equilibrated in starting buffer, 25 mM acetate buffer pH 5.0 and packed in jacketed column (2x50 cm) upto the height of 20 cm. The column was washed with the same buffer system overnight. The dialyzed fraction (60-80 mg) from DEAE cellulose column was applied to the column and the unabsorbed protein was first eluted with 200 ml of 25 mM acetate buffer followed by elution of the absorbed protein through batchwise elution using 450 ml buffer containing NaCl gradient varying from 25 mM to 700 mM. Fractions (2 ml) were collected at a flow rate of 0.4 ml/min. Each fraction was analysed for protein and activity. Tubes containing individual peaks of enzyme activity were pooled, protein was precipitated at 100 % $(NH_4)_2 SO_4$ saturation and centrifuged as before. The precipitate was dissolved in 3-5 ml buffer and dialyzed overnight against the same buffer.

3.2.2.6 Test for Homogeneity by Polyacrylamide Gel Electrophoresis

The homogeneity of the purified enzyme fractions was tested by polyacrylamide gel electrophoresis (PAGE) using 7.5 % polyacrylamide gel in the separating gel columns, in 0.1 M sodium phosphate buffer, pH 7.2. The electrophoresis was carried out according to method described by Davis(172).

3.2.2.6.1 Preparation of Polyacrylamide Gel Columns

The 7.5 % acrylamide gel solution (20 ml) was prepared by mixing 30.0 % acrylamide solution containing 1.0 %, N,N' methylene bis acrylamide (5 ml), 0.2M sodium phosphate buffer, pH 7.2, (14 ml), and 0.35 % ammonium persulphate (1 ml). The combined solution was then deaerated to remove the dissolved air. To the deaerated solution 0.032 ml of TMED (N,N,N',N'- tetraethylene diamine) was added, mixed well and immediately pipetted upto a fixed height into the gel glass tubes, fixed in a vertical position with lower end closed. A drop of buffer was also added on the top of the gelling solution to avoid the formation of concave meniscus.

3.2.2.6.2 Electrophoresis

The tubes with gel columns were fixed in the electrophoretic apparatus and the buffer, 0.1M sodium phosphate (pH 7.2) was filled in the anode and cathod chambers of the apparatus. Individual protein samples (40-80 ug) in 40 % sucrose solutions were layered on the top of seperate gel

columns. To one of the columns 0.05 % bromophenol in 40 % sucrose solution was also applied. The electrophoresis was carried out at a constant current of 5 mA per tube for 2.5 hours or until the tracker dye, bromophenol blue, migrated to the lower end of the gel column. The protein bands were located with the help of anidoblack. Destaining was done in 7.5 % acetic acid solution.

3.3 RESULTS

3.3.1 Separation of Multimolecular Forms of Phytase

Fig. 3 shows the stepwise flow diagram for the isolation of phytase isoenzymes. The results of isolation and purification of various forms of phytase extracted from 7 day germinated Cucurbita maxima seeds are summarized in the Table 1. The purification scheme involved six main steps in the following sequence (i) Acetone powder preparation (ii) Extraction of phytase enzyme from acetone powder (iii) $(NH_4)_2SO_4$ fractionation (iv) Sephadex G-150 gel filtration (v) DEAE cellulose chromatography and (vi) CM-cellulose column chromatography.

The acetone powder preparation was an essential preliminary treatment. It removed the lipids, pigments and other such materials which usually interfere in the isolation of enzymes. In addition, the acetone powder can be stored easily for several months in freezer in an air tight polyethylene bag without any loss in enzyme activity. Purification of enzyme was done 1.1 fold at this stage.

The $(NH_4)_2SO_4$ fraction precipitating between 0.3 and 0.8 saturation contained most of the enzyme. Other fractions showed little or no activity of phytase. At this stage the purification and the yield of phytase were 1.72 and 99 %. respectively. This fraction was subjected to the gel filtration on Sephadex G-150 gel column. Fig. 4 shows the elution profile of phytase enzyme from the column. Two major peaks, peak I and peak II, containing phytase activity were obtained. Peak I which was eluted first contained the high molecular weight phytase species (phytase I) and the peak II which eluted little later contained the low molecular phytase species (phytase II). The high and low molecular weight species accounted for approximately three fourth and one-third of the total phytase activity. There is also present a small peak III with less than 5 %. of the total phytase activity in seeds. This fraction because of the low activity was not processed further. The purification of phytase I and II was 3.8 and 2.7 folds, respectively.

3.3.2 Further Separation and Purification of Different Forms of Phytase I

The phytase I fraction from the Sephadex G-150 column which accounted nearly three fourth of the total phytase activity in sample, was subjected to DEAE-cellulose and CM-cellulose column chromatography for further purification and separation of different isoenzymes of the high molecular phytase. Fig. 5 shows the elution profile of phytase I from the DEAE cellulose column at pH 7.2 One major

peak containing nearly all the phytase activity followed by a relatively small peak with less than 2 % phytase activity were obtained at 0.2 M and 0.3 M NaCl gradient, respectively. The small peak corresponds to the phytase II, the low molecular weight species, indicates presence of contamination since it was eluted exactly at the same salt gradient from the DEAE-cellulose column (Fig. 7). The purification of phytase I at this stage was 8.2 folds with a yield of 70 %.

Phytase I fraction after the DEAE-cellulose column was chromatographed on a CM-cellulose column using stepwise elution with equilibrating buffer containing varying NaCl concentrations (0-700 mM). The elution profile is shown in Fig. 5. Five distinct peaks showing phytase activity were eluted with 50 mM, 200 mM, 300mM, 400 mM and 700 mM NaCl gradient at pH 5.0. These fractions were designated as phytase IA, phytase IB, phytase IC, phytase ID and phytase IE, respectively. The purification of phytase IA, IB, IC, ID, and IE was 34.1, 42.6, 21.3, 16.9 and 25.6 folds with percent yields of 28, 2, 8, 3 and 13, respectively. As will be shown latter these fractions were found to be homogeneous by polyacrylamide gel electrophoresis.

3.3.3 Further Purification of Phytase II

The protein fraction containing the low molecular weight species of phytase, obtained after Sephadex G-150 gel filtration step, was purified further to homogenity by ion-exchange chromatography on DEAE cellulose followed by CM-cellulose using stepwise NaCl gradient elution. The elution profiles are shown in

Figs. 7 and 8 respectively. From the DEAE-cellulose column the phytase activity emerged in a single peak eluting at 300 mM NaCl gradient. The overall purification fold and percent yield based on the crude extract were found to be 2.1 and 20 respectively. The protein peaks eluted at other NaCl gradients did not show any phytase activity. The CM-cellulose chromatography of the phytase II fraction after DEAE-cellulose yielded a sharp peak at 150 mM NaCl which contained the phytase activity (Fig. 8) with a purification of 25.6 folds and yield of 8 %.

3.3.4 Test for Homogeneity

All the six protein fractions containing phytase activity (phytase IA, IB, IC, ID, IE and phytase II) were examined by polyacrylamide gel electrophoresis. A single band was obtained in each case indicating that these enzyme preparations were homogeneous (Fig. 9).

3.4 DISCUSSION

Phytase is currently accepted as an enzyme responsible for the complete hydrolysis of myoinositol hexaphosphate (phytate) to yield myoinositol and orthophosphate as intermediary products (23,26,29). This, however, raises an interesting question, i.e. whether the complete hydrolysis of phytate *in vivo* is carried out by a single phytase or multiple phytase forms (isoenzymes) are involved which carry out the process to completion by acting on different myoinositol phosphate intermediates produced during the enzymic hydrolysis

in a selective manner. Although there is no clear cut evidence, the existence of several types of phytase differing with their origin (74-76,151) and multiple forms of the enzyme in the same plants favour the latter hypothesis. So far in most cases the phytase preparations used were not homogeneous and the only previous report in which five different forms of phytase were isolated from 6 day old pumpkin cotyledons was published by Goel and Sharma (151). The yield of different phytase species was quite low and the losses were too great to be overlooked. Nevertheless, their results were significant for further research in this direction.

The scheme presented in Fig. 3 was modified to give improved yields. The major modifications introduced were as follows: (i) Sephadex G-150 instead of Sephadex G-100 was used to separate the high and low molecular weight phytase forms. This not only gave better separation, but also the yields were quite high, especially the yield of low molecular weight phytase (phytase II) increase from merely 6 % to 22 %., (ii) the ion exchange column chromatography with both DEAE cellulose and CM-cellulose was performed with stepwise gradient using a predetermined narrow range of NaCl gradient (determined by linear NaCl gradient). This concentrated the enzyme in relatively narrow peaks and a base line separation was achieved giving homogeneous phytase fractions as analyzed by polyacrylamide gel electrophoresis. The yields for phytase I and phytase II improved by a factor of 2 and 3.5, respectively.

Besides improved yield, an additional phytase form

(phytase IE) was eluted from the CM-cellulose column at 700 mM NaCl gradient which was not reported by Goel and Sharma as these workers used a salt gradient upto 500 mM. This additional phytase I species accounts for nearly one-fourth of the combined phytase I isoenzymes activity recovered from the CM-cellulose column and about 13 % of the total phytase activity in cotyledons. Thus, it is expected to contribute sufficiently in achieving the complete dephosphorylation of phytate molecule. This aspect will be discussed in latter chapters.

The separation and purification of six different forms of phytase enzyme from the germinating pumpkin cotyledons has provided an excellent system to study the specific physiological role of various forms of phytase in the seed metabolism during germination and early period of plant growth and also to elucidate the mechanism of phytate degradation in vivo.

3.5 SUMMARY

Multimolecular forms of phytase (myoinositol hexaphosphate phosphohydrolase, EC 3.1.3.8) were isolated and purified from germinating cotyledons of Cucurbita maxima using acetone treatment, ammonium sulphate fractionation, Sephadex gel filtration and ion exchange chromatography of DEAE and CM-cellulose columns. By Sephadex gel filtration two main forms of phytase were isolated high molecular weight (Phytase I) and low molecular weight (Phytase II). The Phytase I was

further resolved into five distinct forms on CM cellulose which were designated as phytase IA., IB, IC, ID and IE. On the other hand phytase II was eluted as a single species from CM cellulose column. The results of polyacrylamide gel electrophoresis of purified forms of phytase showed that all of them were purified upto homogeneity.

TABLE-1 : PURIFICATION TABLE OF VARIOUS PHYTASE FORMS

Sl. No.	Purification step	Total protein (mg)	Total units	Sp.Act. (units/mg)	% yield[1]	Purification fold[2]
1.	Crude extract	1034	3977	3.90	100	
2.	Acetone powder extract	955	3979	4.12	100	1.06
3.	$(NH_4)_2SO_4$ precipitation					
	(i) 0.3 saturation	355	-	-	-	-
	(ii) 0.8 saturation	588	3920	6.70	99	1.72
4.	Sephadex G-150					
	Peak I(Phytase I)	197	2906	14.70	73	3.77
	Peak II(Phytase II)	83	860	10.40	22	2.67
5.	DEAE-cellulose of Phytase I Frac.	86	2768	32.0	70	8.21
6.	CM cellulose of Phytase I					
	Peak I (Phytase IA)	9.0	1125	133	28	34.10
	Peak II (Phytase IB)	0.5	83	166	2	42.56
	Peak III(Phytase IC)	3.6	300	83	8	21.28
	Peak IV(Phytase ID)	1.8	120	66	3	16.92
	Peak V(Phytase IE)	5.1	510	100	13	25.64
7.	DEAE Cellulose of Phytase II Fraction	67	812	12.0	20	3.08
8.	CM-Cellulose of Phytase II	7.2	720	100	18	25.64

1. % Yield = $\frac{\text{Total units of purification stop}}{\text{Total units of crude extract}} \times 100$

2. Purification fold = $\frac{\text{Specific activity of purification step}}{\text{Specific activity of crude extract}}$

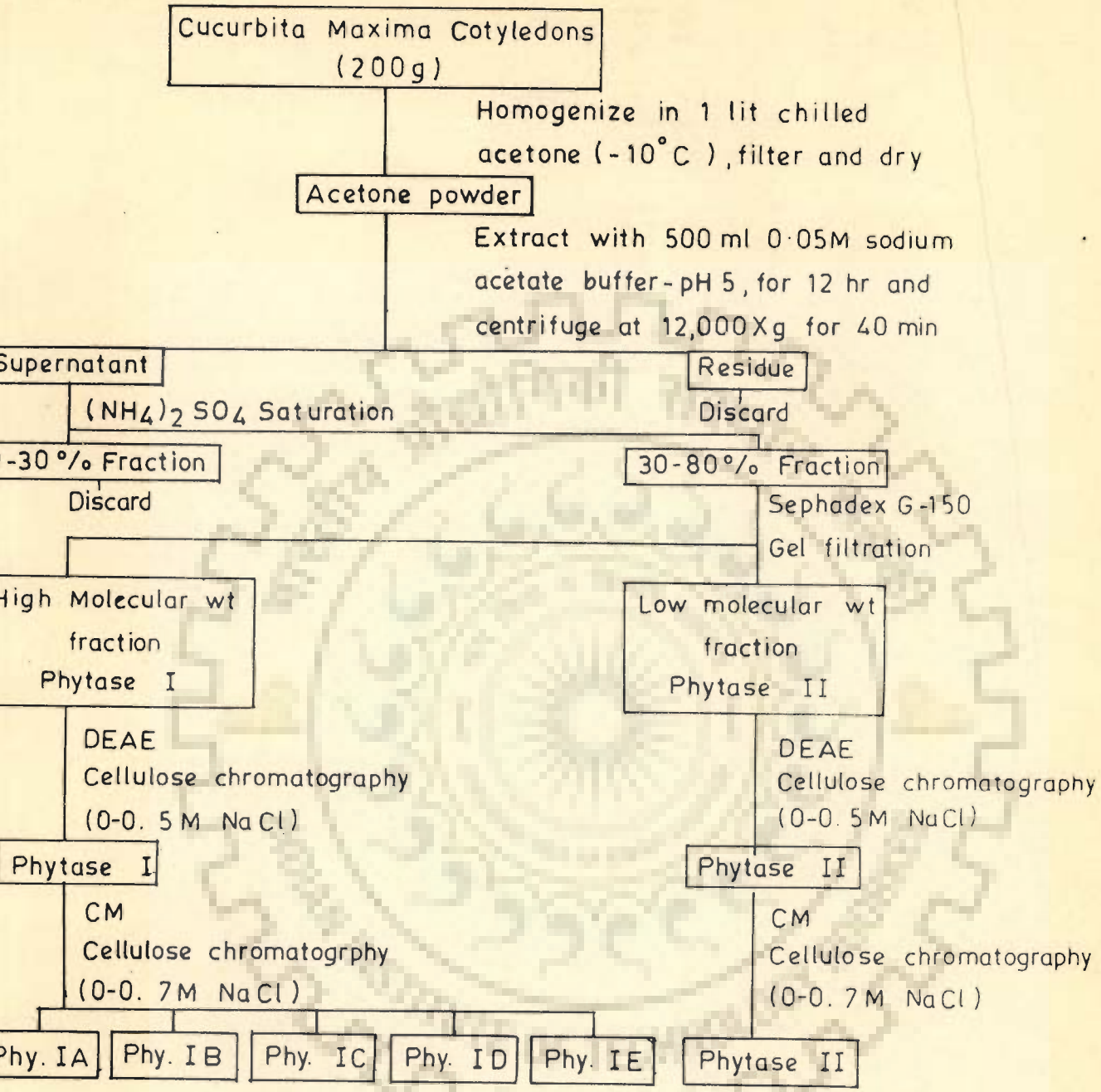


Fig. 3 - Purification Scheme for Isolation of Multimolecuar Forms of Phytase

Fig. 4 : SEPARATION OF HIGH MOLECULAR AND LOW MOLECULAR FORMS OF PHYTASE BY SEPHADEX G-150 GEL FILTRATION.

Gel filtration of $(NH_4)_2 SO_4$ fraction (0.3-0.8 saturation) was carried out on a Sephadex G-150 column (1 x 70 cm). The elution was done by 0.05M acetate buffer (pH5). Two ml fractions were collected at a flow rate of 6 ml/hr. Enzyme activity and protein concentration were measured in 0.1 ml of each fraction as optical density (O.D.) of the released inorganic phosphate (P_i) at 690 nm and of protein at 660 nm wave length. Optical densities of P_i and protein are shown as -O-O- and -●●- in the figure.

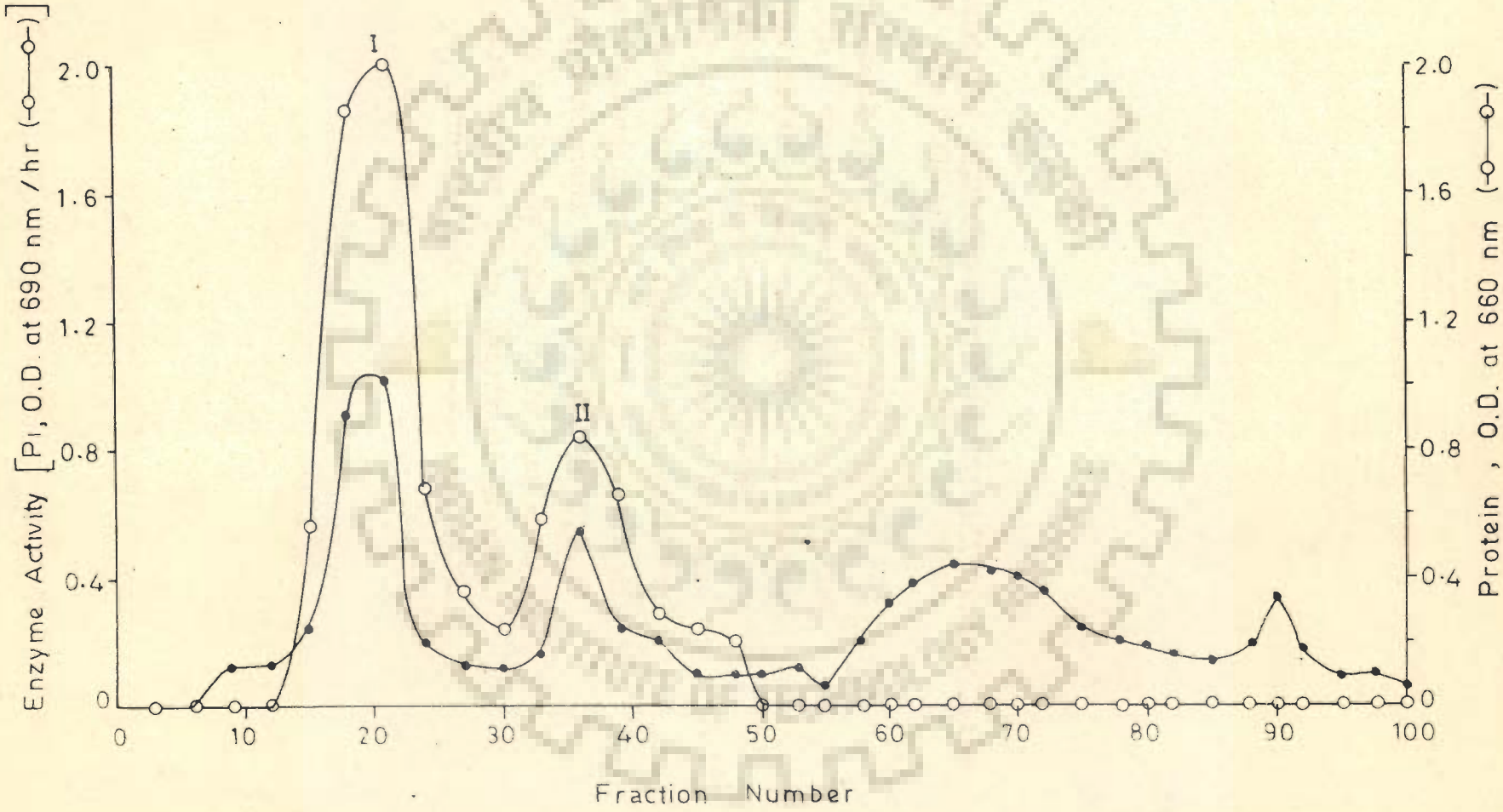


Fig 4 - Gel Filtration on Sephadex G - 150

Fig. 5 : DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PHYTASE I

The (high molecular weight) phytase I fraction obtained after the Sephadex G-150 gel filtration was chromatographed on DEAE-cellulose column (2 x 25 cm) by stepwise elution with 25 mM Tris-HCl buffer (pH 7.2) containing increasing NaCl concentration (0-500 mM). Two ml fractions were collected at a flow rate of 0.4 ml/min. Enzyme activity and protein concentration were measured in 0.1 ml of each fraction as optical density of the released inorganic phosphate (P_i) at 690 nm and of protein at 660 nm. Optical densities of P_i and protein are represented as -O-O and -●-● in the figure.

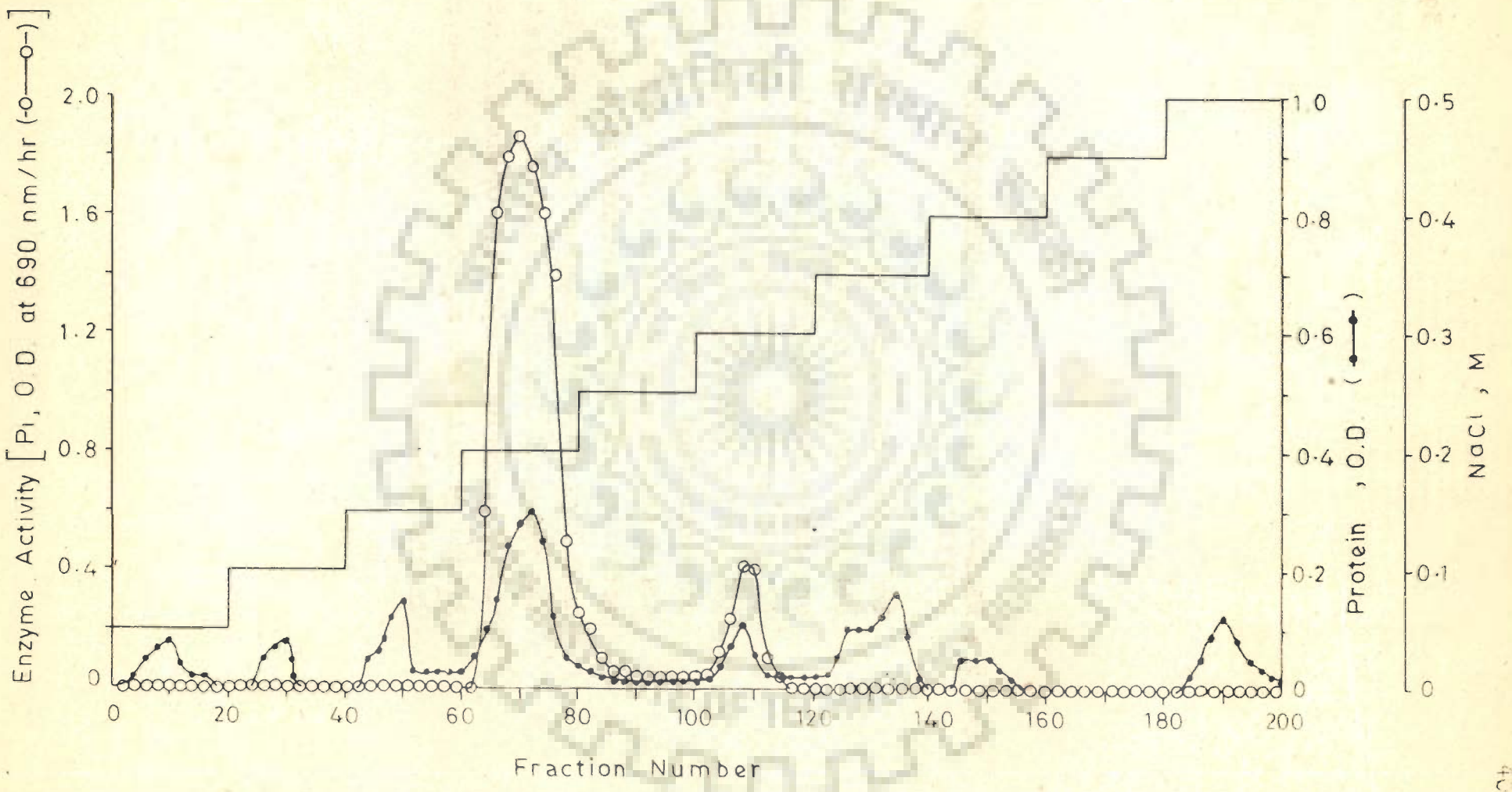


Fig 5 - DEAE Cellulose Column Chromatography of Pnytase I

Fig. 6 : CM-CELLULOSE COLUMN CHROMATOGRAPHY OF PHYTASE I.

Phytase I fraction from DEAE cellulose column was further chromatographed on CM-cellulose column (2 x 20 cm). The batchwise elution was done by increasing concentration of NaCl (0-700 mM) in 25 mM sodium acetate buffer, pH5. Two ml fractions were collected at a flow rate of 0.4 ml/min. Enzyme activity and protein concentration were measured in 0.1 ml of each fraction as optical density of inorganic phosphate (released at 690 nm)and of protein at 660 nm. Optical densities of P_i and protein are represented as -O-O and -●●- in figure.

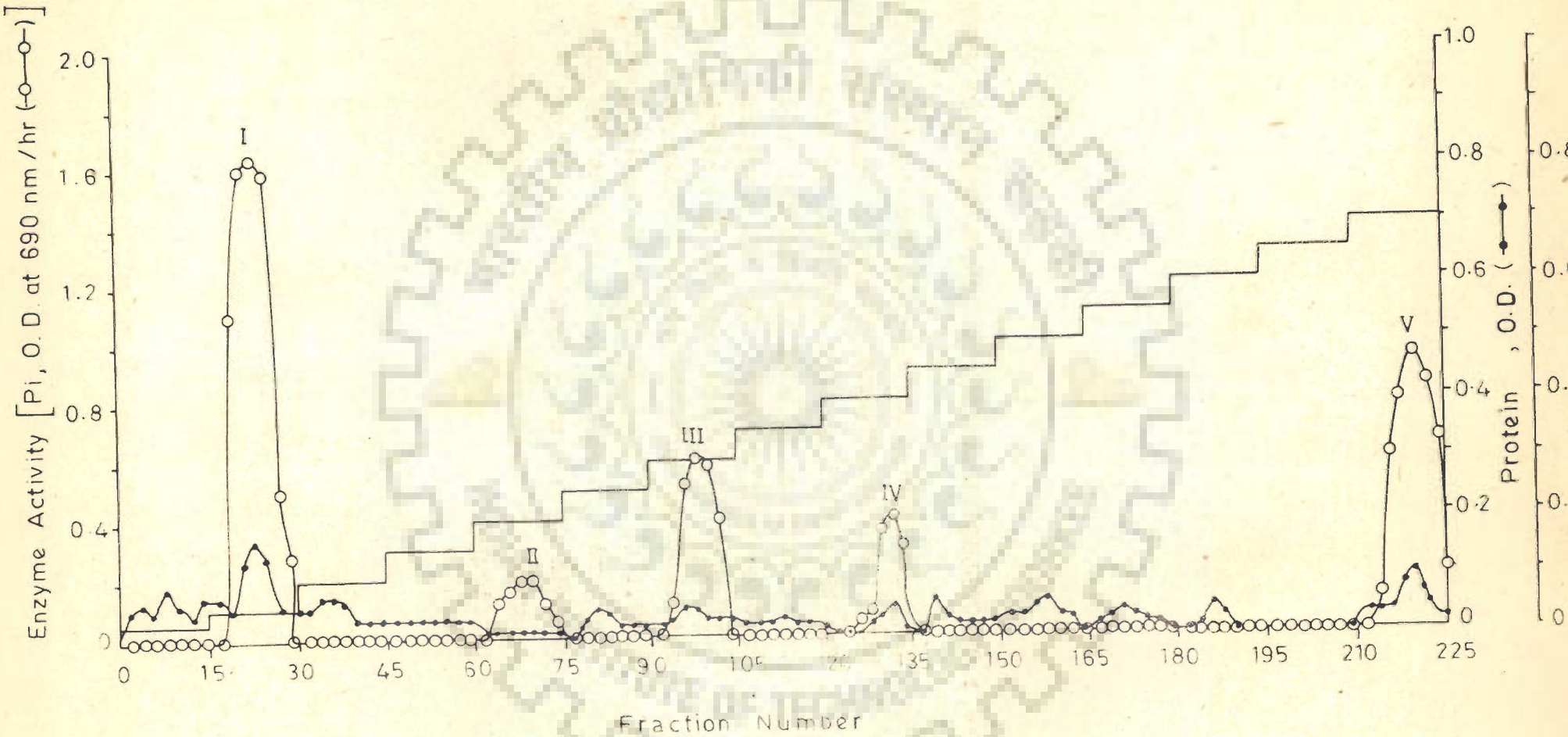


Fig. 6 - CM Cellulose Column Chromatography of Phytase I

Fig. 7 : DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PHYTASE II.

Chromatography of phytase II peak, eluted from Sephadex G-150 column, was carried out on a DEAE cellulose column (2 x 25 cm). The batchwise elution was done by increasing concentrations (0 to 500 mM) of NaCl solution in 0.025 M tris-HCl buffer (pH 7.2). 2 ml fractions were collected at a flow rate of 0.4 ml/min. Enzyme activity and protein concentration were determined in 0.1ml of each fraction as optical density of the released inorganic phosphate (P_i) at 690 nm and of protein at 660 nm. Optical densities of P_i and protein are represented as -O-O- and -●-● in figure.

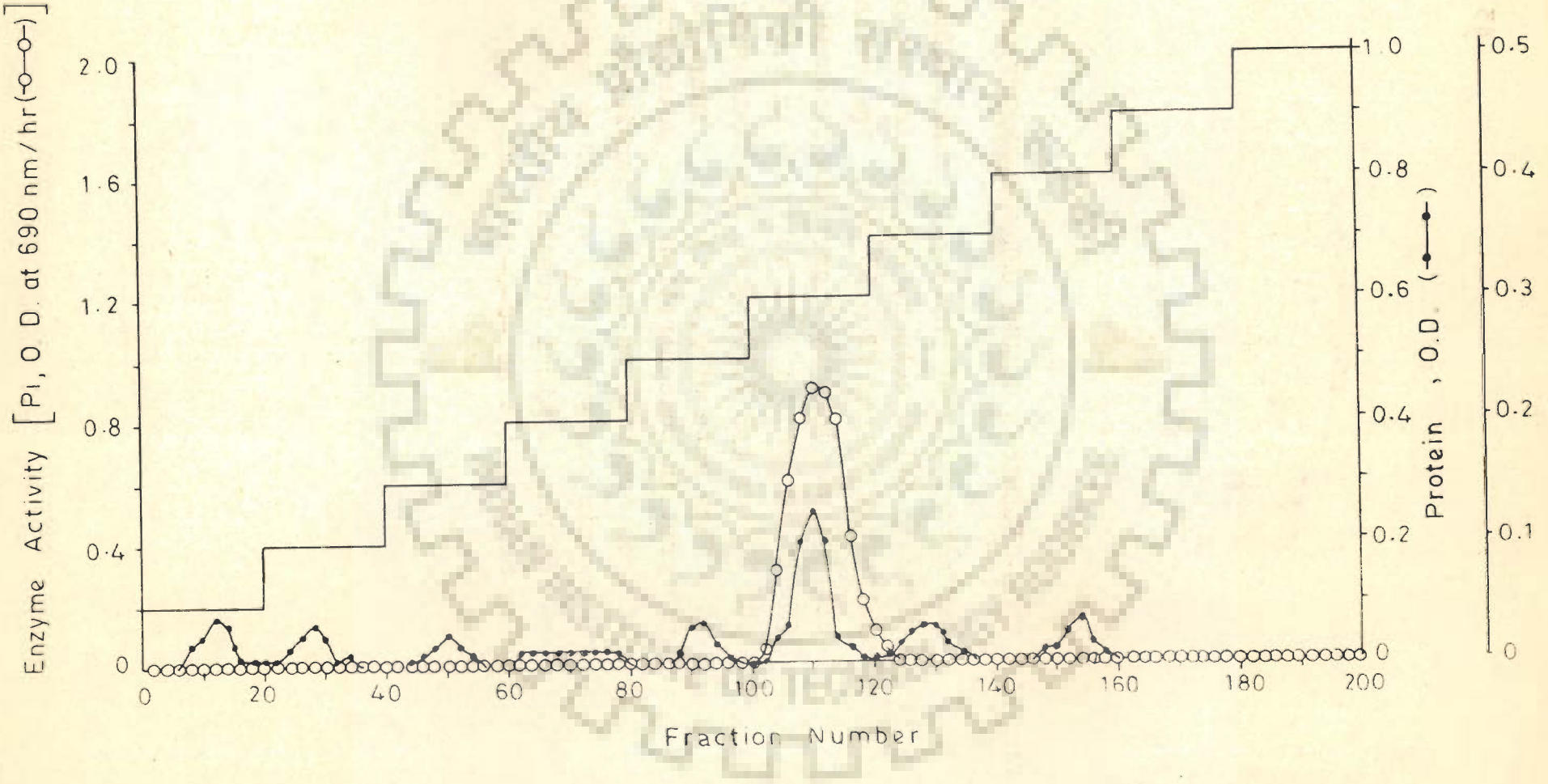


Fig. 7 - DEAE Cellulose Column Chromatography of Phytase II

Fig. 8 : CM-CELLULOSE COLUMN CHROMATOGRAPHY OF PHYTASE II.

Phytase II fraction from DEAE cellulose column was further chromatographed on CM-cellulose column (2 x 20 cm). The batchwise elution was done by increasing NaCl concentration (0 - 700 mM) in 25 nM sodium acetate buffer, pH5. Two ml fractions were collected at a flow rate of 0.4 ml/min. Enzyme activity and protein concentration were determined in 0.1 ml of each fraction as optical density of the released inorganic phosphate (P_i) at 690 nm and of protein at 660 nm. Optical densities of P_i and protein are represented as -O-O- and -●●- in figure.

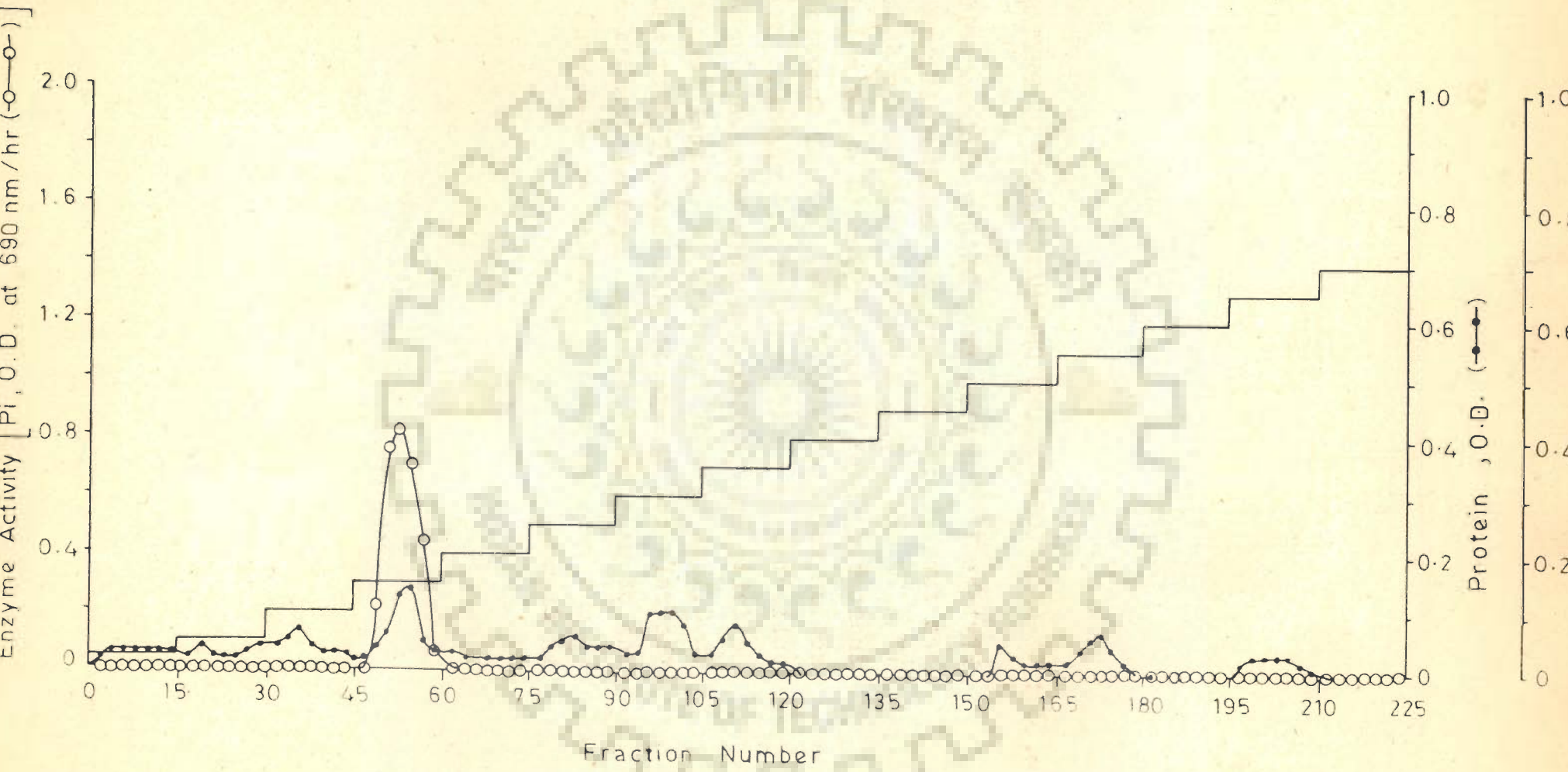
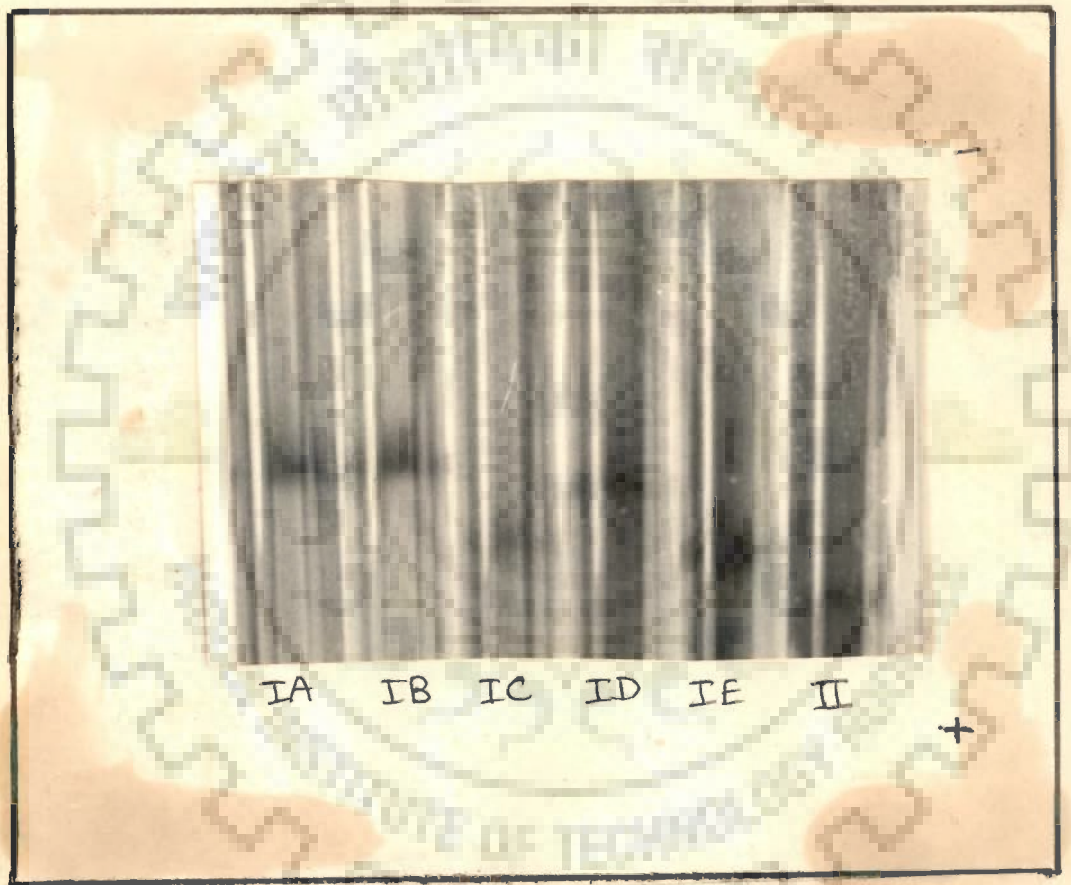


Fig 8 - CM Cellulose Column Chromatography of Phytase II

Fig. 9 : POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS OF MULTIMOLECULAR FORMS OF *CUCURBITA MAXIMA* PHYTASE.

The electrophoresis was carried out on 7.5 %. polyacrylamide gel columns in 0.10 M phosphate buffer, pH 7.2, using bromophenol blue as the tracker dye. A current of 5 mA per tube was passed constantly for 150 min. The protein bands were located by amido black. The detailed procedure is given in the text.



Electrophoretic Patterns of Phytase Isoenzymes

CHAPTER-IV

CHARACTERIZATION OF MULTIMOLECULAR SPECIES OF PHYTASE FROM THE SEEDS OF CUCURBITA MAXIMA

4.1 INTRODUCTION

In the previous chapter separation and purification of six molecular forms of phytase were covered. In this chapter properties of these enzymes have been investigated in details.

4.2 MATERIALS AND METHODS

4.2.1 Materials

All the chemicals used were of Analytical Reagent(AR) grade. Sodium phytate was obtained from the same source as described earlier. Phytase isoenzymes were extracted and purified from 7 day old germinating cotyledons of Cucurbita maxima seeds according to the scheme outlined in Chapter-III. Various myoinositol phosphates were obtained from hydrolysis products of myoinositol hexaphosphate by phytase enzyme of Cucurbita maxima as described below. The homogeneity of the myoinositol phsophates was checked by paper chromatography(91) and the myoinositol-phosphate ratio of the purified fractions was determined by the method of Cosgrove(173). In a typical experiment 5 g sodium phytate was dissolved in 100-125 ml of 0.1 M sodium acetate buffer of pH 4.8. The final pH of the resultant solution which increased by dissolving sodium phytate, was adjusted by titrating it with 10 M acetic acid. Finally the volume was made upto 250 ml with 0.05 M acetate

buffer to obtain 0.025 M phytate solution. To this sufficient amount of partially purified enzyme (15 mg/ml) from pumpkin seeds was added and incubation was carried out at 40°C until nearly 50% of the phytate phosphorus was hydrolyzed by the enzyme. The progress of the phytic acid hydrolysis was followed by measuring the amount of P_i released, by the method of Fiske Subba Row(152) at different intervals. After the desired hydrolysis was achieved, the enzyme action was stopped by heating the tubes in boiling water for 15 min. The contents were allowed to cool and then extracted with 20% trichloroacetic acid. The denatured protein was removed by centrifuging at 12,000 Xg for 30 min and the clear supernatant was decanted off. The pH of the supernatant was adjusted to 8.0 with 10 NaOH and the myo-inositol phosphates were precipitated as barium salts by the addition of excess of barium acetate as described by Sharma and Dieckert(174). The precipitate was separated by centrifugation. The supernatant was concentrated to about 50 ml at 40°C under reduced pressure and the barium salt of inositol monophosphate was precipitated by adding 4 vol of ethanol(174) or by lead acetate (68). The barium salts of myoinositol phosphates were dissolved in minimal volume of 3 N HCl and centrifuged to remove any isoluble material. The clear supernatant was readjusted to pH 8 with 10 N NaOH and the barium salts of myoinositol phosphates were precipitated by the addition of excess of barium acetate. The precipitate was separated by centrifugation. The supernatant was concentrated to about 50 ml at 40°C under reduced

pressure and the Ba-salt of inositol monophosphate was precipitated by adding 4 vol of ethanol or by adding lead acetate. The clear supernatant was readjusted to pH 8 with 10 N NaOH and the barium salts of myoinositol phosphates were precipitated as before. Myoinositol monophosphate fraction was treated separately. The barium salts of myoinositol phosphates were decomposed with Dowex-50x8 (H^+), 50 mesh, to obtain myoinositol phosphates in solution in the free acid form for chromatographic examination.

4.2.1.1 Ion Exchange Chromatography of Myoinositol Phosphates

Myoinositol phosphates obtained above were chromatographed on a Dowex -1x8 (Cl^-) , 400 mesh, column (1x50cm) by linear gradient elution with 0-1 M NaCl as described by Grado and Ballou(175). In gradient system the reservoir and the mixing chamber contained 1 lit. of 1 M NaCl and 1 lit of water. The rate of elution was 1 ml per min. 10 ml fractions were collected and analyzed for total P by the method of Bartlett(176). Fractions of a given phosphorus peak represented a particular myoinositol phosphate ester being separated by Dowex- 1x8 (Cl^-) column chromatography. The fraction of each peak were pooled and barium salts of various myoinositol phosphates including the hexaphosphate were precipitated as mentioned in the preceding section. The insoluble salts were collected by centrifugation and ionized with Dowex-50 (H^+). Various myoinositol phosphate esters present in these fractions were further identified by paper chromatography.

4.2.1.2 Paper Chromatography of Myoinositol Phosphates

For identification and purification of various myoinositol phosphates, obtained from the Dowex-1(Cl^-) column, ascending paper chromatography was carried out on Whatman No.1 filter paper using 2-propanol-NH_3 - H_2O (5:4:1) as the solvent system(91). The paper chromatogram containing a standard mixture of lower myoinositol phosphates and individual fraction from the column was developed in this solvent system for 48 hours at 30°C in a chromatographic chamber fully equilibrated with the vapours of the same solvent. After the chromatogram was fully developed, it was removed from the chamber and dried in air. In order to locate the myoinositol phosphate esters, the chromatogram was sprayed with Axelrod and Bandurski reagent (177) prepared by mixing 45 ml of $HClO_4$ (70% w/v), 10 ml of conc. HCl, 1.0 g EDTA sodium salt and 945 ml of 1% ammonium molybdate. The chromatogram was dried in air, heated for 2 min at 85°C in a hot air oven and then exposed to short wave length U.V. light for 20-30 min. The blue spots were formed indicating the presence of myoinositol phosphate esters. Guide strips were cut and the P containing bands were located. The unsprayed portions of the chromatogram with myoinositol phosphates were cut and the compounds were eluted with H_2O. The eluates were neutralized with ammonium hydroxide and concentrated to about 5 ml at 40°C with the help of a rotary evaporator.

4.2.2 Methods

Enzyme assay was done in the same way as described in Chapter-III. The effect of pH on the activity of various isoenzymes was studied by assaying the enzyme activity at different pH ranging from 3.6 to 7.0. The buffers used were sodium acetate buffer. The pH of buffers was adjusted by the addition of NaOH. The results are expressed as percent of maximum activity versus pH.

4.2.2.1 Determination of Thermal Stability

The thermal stability of the isoenzymes was determined by preincubating the enzyme solution without substrate in 0.1M acetate buffer of optimum pH at indicated temperatures varying from 20° to 80°C for 60 min. After incubation enzymes were then quickly cooled in ice bath to arrest the thermal denaturation state of the enzyme and then assayed in a standard way at 40°C as described before. The activity in the control (without preincubation) was used as 100 percent and the results are expressed as the percent residual activity as a function of temperature.

4.2.2.2 Kinetic Studies

In all the experiments described in this section, conditions of incubation were same as described under enzyme assay unless otherwise stated.

4.2.2.2.1 Determination of Apparent Michaelis Menten Constant(K_m) and Maximum Velocity (V_{max}) by Line Weaver Burk Plots

The values of K_m and V_{max} for each of the phytase isoenzymes were evaluated from Lineweaver Burk plots(156). Measurement of enzyme activity for these plots was made at optimum pH, with different substrate concentrations varying from 0.01 mM to 2 mM for each plot. Reaction velocity at different substrate concentrations was determined by measuring the amount of phosphate liberated per unit time. The double reciprocal plots of velocity versus substrate concentrations were plotted on linear graph paper. The intercepts on the 1/S axis and on the 1/V axis were equal to $-1/K_m$ and $1/V_{max}$, respectively.

4.2.2.2.2 Apparent Number of Substrate Molecules (n) Interacting per Molecule of Phytase Enzyme

The number of substrate molecules 'n' interacting with one molecule of the enzyme, was determined by the method of Hill(157) by plotting the data according to the following equation:

$$\text{Log}\left(\frac{V}{V_{max}-V}\right) = \log 1/K_s + n \log(S)$$

where, V_{max} being the maximum velocity , V the velocity at any substrate concentration (S), K_s is the binding constant for substrate and 'n' the interaction constant.

Plots of log (S) against log ($V/V_{max}-V$) showed linear relationship. The value of 'n' was computed from

the slope of the curve as in these plots the slope directly eqhals the interaction constant (n).

4.2.2.2.3 Determination of Thermodynamic Parameters

Experiments concerning thermodynamic studies were carried out in usual manner. The thermodynamic parameters, Ea, ΔH, ΔG and ΔS were determined by assaying the enzyme activity at various temperatures (30°-$50^{\circ}C$). The methods are given below.

4.2.2.2.3.1 Energy of Activation (Ea)

The energy of activation for the phytase catalyzed reactions was determined from Arrhenius plots (158) according to following equation:

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

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 equations:

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

4.2.2.2.3.2 Determination of Enthalpy Change(ΔH)

The value of ΔH for the hydrolysis of phytic acid by phytase isoenzymes was determined by the following

Vant Hoff's equation:

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

where, A is a constant factor related to moleuclar 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 1/T will be linear with a slope of $-\Delta H/2.303R$ from which the value of ΔH was calculated.

4.2.2.2.3.3 Determination of Free Energy Change (ΔG) and Entropy Change (ΔS)

The thermodynamic parameters, ΔG and ΔS were calculated by using following equations:

$$\Delta G = -2.303\ RT \log K_m$$

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

The terms in these equations have their usual meanings.

4.2.3 Determination of Electrophoretic Mobility and Molecular Weight by Gel Electrophoresis

For the determination of electrophoretic mobility, the polyacrylamide gel electrophoresis was carried out as described in section 3.2.2.6 of Chapter-III unless stated otherwise. The electrophoretic mobility with respect to bro-

mophenol blue was measured by the following equation:

$$E_m = \frac{X_p \times \ell}{X_b \times \ell'}$$

where,

E_m = Electrophoretic mobility ratio

X_p = Distance of protein migration from origin

X_b = Distance of bromophenol migration from origin.

and ℓ and ℓ' are gel lengths before and after staining, respectively.

The molecular weights of phytase-isoenzymes were determined by SDS Poly Acrylamide Gel Electrophoresis (SDS-PAGE). For this purpose a curve between the molecular weight and the electrophoretic mobility(EM) was plotted by employing molecular weights of different protein standards supplied by Bio Rad Labs., Richmond, California. The molecular weights of different standard proteins used were as follows:

Protein	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
β Galactosidase	1,16,250
Myosin	2,00,000

In these studies this curve was used as a standard calibra-

tion curve for determining the values of molecular weights corresponding to the measured EMs of different phytase isoenzymes. See Fig.17.

SDS-Polyacrylamide gel electrophoresis was carried out using 12.6% and 4% concentration of polyacrylamide as separating and stacking gels, respectively, in 0.1M sodium phosphate buffer, pH 8.3 containing 0.3M glycine and 0.1% SDS (155).

4.2.3.1 Preparation of Polyacrylamide Gel Columns

The 12.6% polyacrylamide separating gel solution (20 ml) was prepared by mixing 25.2% acrylamide solution containing 0.8% N,N' methylene bis acrylamide (10 ml), 0.2M sodium phosphate buffer, pH 8.8, containing 0.1% SDS (9 ml) and 0.35% ammonium persulphate (1 ml). The combined solution was then deaerated to remove the dissolved air. To the deaerated solution TMED (N,N,N',N' tetraethylene diamine) (0.092 ml) was added, mixed well and immediately pipetted into the gel glass tubes, fixed in a vertical position with lower end closed, upto a fixed height so as to give gel columns of 10 cm height. A drop of buffer was also added on the top of the gelling solution. A small stacking gel column (1 cm long) was parepared in the same way using only 4.0% acrylamide gel in 0.1M sodium phosphate buffer, pH 7.2.

4.2.3.2 Preparation of Protein Sample for Electrophoresis

50 μg of protein sample was heated at 100^{o}C for

5 minutes. After cooling at room temperature it was diluted to 1:20 (v/v) by the diluting buffer of the following composition: Sodium phosphate buffer, pH 7, 10mM; SDS, 1%; -mercaptoethanol, 100 mM; sucrose, 40%; and bromophenol, 0.001%.

4.2.3.3 Electrophoresis

The tubes with gel columns were fixed in the electrophoretic apparatus and the buffer, 0.1M sodium phosphate (pH 8.3) containing 0.1% SDS and 0.3M glycine was filled in the electrode chambers of the apparatus.

The electrophoresis was carried out in the same way as described in section 3.2.2.6.2 of Chapter-III.

4.2.4 Substrate Specificity Test of Phytase Isoenzymes Towards Different Myoinositol Polyphosphate Intermediates Produced During the PhytateHydrolysis by a Mixed Phytase Enzyme System

The specificity of the different purified phytase species was tested using mono-, di-, tri-, tetra-, penta- and hexaphosphate esters of myoinositol. The experimental conditions for the enzyme assay were those described under Methods and Materials section of Chapter- III except that the pH of incubation for each form of phytase was corresponding to its pH optimum. The results are based on the ratio of V_{max} /K_m.

4.3 RESULTS

4.3.1 Effect of pH

The effect of pH on the activity of different mole-

cular species of phytase was studied by using phytic acid as substrate. The pH-activity plots are shown in Fig 10. All the pH activity curves for phytase IA, IB, IC, ID, IE and phytase II are sharply peaked at pH 5.0, 4.8, 5.0, 5.2, 4.4, and 5.6, respectively. These results indicate the acid phosphatase nature of enzymes operating in a fairly wide acid pH range from pH 4.4 to 5.6. This property may be of physiological significance as will be shown in latter chapters that the low molecular weight form of phytase (phytase II) which is dominant in the later stages of germination shows the highest optimum pH. In contrast the phytase IE shows the most acidic optimum pH (4.4). This particular enzyme was found to increase continuously during the entire period of germination. The enzyme phytase IA which is another molecular specie is most dominant in the very early period of germination and it has optimum pH of 5.0. A comparison of these results, therefore, indicates that dominant species i.e. phytase IA and phytase IB act nearly in identical pH environment.

4.3.2 Thermal Stability of Phytases

The effect of temperature on the stability of phytases was studied by exposing the different enzymes to various temperatures for a period of 60 minutes and then assaying the residual activity after cooling in ice for half an hour. The results are shown in Fig.11. From these results it is apparent that the various molecular forms of phytase I show 100% stability only upto 40°C. After that

there is a rapid decline in the enzyme activity. The low molecular weight enzyme phytase II shows the highest thermal stability showing 100% stability upto 50°C and after 1 hr exposure at 70°, this enzyme still shows a residual activity of nearly 70% whereas the residual activity for high molecular weight phytase species (IA to IE) varies between 18 and 44%. In each case the temperature at which half of the activity remains, differs significantly, indicating the distinct structural differences.

4.3.3 Molecular Weight Determination of Multimolecular Species of Phytase

The molecular weights of various forms of phytases were determined by SDS PAGE using the following standard proteins: lysozyme (14,400 Daltons), soyabean trypsin inhibitor (21,500 Daltons), carbonic anhydrase (31,000 Daltons), ovalbumin (45,000 Daltons), BSA (66,200 Daltons), phosphorylase B (92,500 Daltons), β galactosidase (1,16,250 Daltons), myosin (2,00,000 Daltons). The calibration curve for the molecular weight determination is shown in Fig.17. The molecular weight of the phytase species (IA, IB, IC, ID, IE and II) were found to be 60,256; 63,096; 43,657; 77,625; 38,905 and 29,512 Daltons respectively.

There is large variation in the molecular weights of different species of phytase. The lowest being 29,512 for phytase II and highest for the phytase ID species. The molecular weights of IA and IB are very close to one another but of the remainder species are significantly different.

The electrophoretic mobility ratio of various phytase species relative to bromophenol blue at pH 7.2 in 0.1M phosphate buffer are given in Table.6. It was observed that phytase II shows the highest electrophoretic mobility ratio i,e. 0.69 and phytase IB shows the lowest i.e. 0.48.

4.3.4 Kinetic Studies

4.3.4.1 Determination of K_m and V_{max}

The values for apparent K_m and V_{max} of various enzymes with phytic acid substrate were determined by Lineweaver Burk plots as shown in Fig.12. These curves were obtained by plotting the reciprocals of velocity obtained at 40°C and optimum pH versus reciprocals of substrate concentrations. The values of apparent K_m for enzyme IA, IB, IC, ID, IE and II were found to be 0.125, 0.227, 0.156, 0.156, 0.052, 0.075 mM respectively and values for V_{max} were found to be 11.8, 33.3, 16.4, 20.0, 11.1, 14.3 mM/hr respectively. On the basis of ratios of V_{max}/K_m it is apparent that phytase II and phytase IE appear to be the most suitable enzyme for the dephosphorylation of phytic acid. This question was however investigated in greater detail under the section 4.3.6 dealing with the results.

4.3.4.2 Apparent Number of Myoinositol Hexaphosphate Molecules 'n' Combining with one Molecule of Phytase Species

The apparent number 'n' of the interacting molecules of the substrate per molecule of phytase isoenzyme were determined from the slope of Hill's plots shown in Fig.14. In each experiment plots of log $V/(V_{max}-V)$ against log(S) showed linear relationship with a constant slope of approximately one indicating that the apparent number of interacting molecules of the substrate per molecule of the enzyme is one. This value remained unaffected by temperature in the temperature range 30°-50°C. These results also indicate that the phenomenon of cooperativity does not occur in the phytase-phytic acid interaction.

4.3.5 Thermodynamic Studies

4.3.5.1 Thermodynamic Parameters

The effect of temperature on the activity of phytase enzymes was further investigated by determining various thermodynamic parameters. In order to avoid the thermal denaturation the temperature range between 30° and 50°C was selected.

The values of temperature quotient, Q_{10}, for enzyme IA, IB, IC, ID, IE & II are 1.3, 1.2, 1.5, 1.3, 2.3 and 2.3. It is of interest to note that the first four species show identical Q_{10} values while the phytase IE and phytase II have same and higher Q_{10} values. If we consider that the molecular size of the enzyme may be contributing significantly

towards the thermal properties of enzyme then the low molecular weight species of IE (38,905) and phytase II(29,512) show greater Q_{10} than those of high molecular weight species.

Fig.15. shows the Arrhenius plots of log V_{max} versus 1/T ^{o}K for various molecular forms of phytase used to determine the energy of activation. The results as computed from these plots are shown in Table 3. The values of Ea are different for each enzyme with low molecular weight enzyme phytase II showing the lowest energy of activation (3,685 Kcal/mole) and enzyme IE with highest energy of activation (6,909 Kcal/mole).

Fig.13. illustrates the linear plots of log K_m versus 1/T for various phytase forms used to determine the values of ΔH. The ΔH values in each case are positive indicating the endo-thermic nature of reaction and again the ΔH value for phytase II was the lowest. Further these results indicate that there are characteristic values of energy of activation and enthalpy change for the interaction between phytate and various phytase species.

Other thermodynamic parameters entropy change (ΔS), free energy change (ΔG) for the interaction of phytases with sodium phytate were determined at 4 different temperatures (30^{o},40^{o},45^{o},$50^{o}C$). The values of ΔG were computed from the following equation in which K_m has been assumed as dissociation constant.

$$\Delta G = -RT \ln K_m$$

Table 4. shows the values of free energy change (ΔG) and entropy change (ΔS) as a function of temperature. The ΔG values for various enzymes vary from 921 Kcal/mole to 1,884 Kcal/mole. The lowest free energy change occurs in the case of phytate hydrolysis catalysed by enzyme IB and the highest value is for enzyme IE followed by enzyme II. In the case of enzyme IA, IC, ID there is a regular decrease in the free energy change with temperature in contrast, there is no or little change in the energy in case of enzyme IB, IE and II.

The ΔS values of various enzymes are significantly different. At 30°C the ΔS values are positive for all forms of enzyme. There is no significant change in ΔS values of various enzymes with change in temperature between 30°-50°C. The constant ΔS values indicate that no significant conformational change occurs in the enzyme active site as a result of heat treatment in the temperature range 30°-50°C. It is interesting, however, that the conformational change as indicated by the ΔS values for various species are not only characteristic but also markedly different. The large differences in ΔS values among various phytase species with phytic acid as substrate indicate that each enzyme undergoes a varying degree of conformational change. The maximum change occurs in the case of enzyme ID. The enzyme IB shows zero change followed by II and IE which show vary small change in ΔS values with change in temperature.

4.3.6 Determination of Substrate Affinity with the Help of Kinetic Parameters

With a view to find out specific role of various molecular forms of phytase, the kinetic parameters for the myoinositol polyphosphate intermediates produced in the dephosphorylation of phytic acid by phytase, were determined. The main approach was to determine the K_m and V_{max} values and on the basis of V_{max}/K_m ratios the most suitable enzyme-substrate complex formation was established.

Fig. 18.1 to 18.4 show the Lineweaver Burk plots for different enzymes using a particular myoinositol polyphosphate as substrate. In each case the linear plots were obtained. The apparent K_m and V_{max} values are summarised in Table 9. The ratio of V_{max}/K_m for various enzymes and inositol phosphates are given in Table 10. If one goes by the order of K_m, then the myoinositol hexaphosphate will be the most suitable substrate for enzyme IE closely followed by enzyme II, both of which are relatively low molecular weight forms of the phytase enzyme. Further the optimum pH for enzyme IE is also lowest. Based on the same consideration myoinositol pentaphosphate is a preferred substrate for phytase II followed by phytase ID, IB and IC. It is noteworthy that pentaphosphate is the least preferred substrate for enzyme IE, whereas the latter's preferred substrate was hexaphosphate. Myoinositol tetra and triphosphates which represent approximately the middle intermediates in the dephosphorylation scheme and which perhaps are also regulatory points of the scheme, seem to

have nearly equal preference for all the forms of phytase except phytase IE form, Such a system points to a situation when the large concentrations of myoinositol triphosphate and tetraphosphate accumulate and all the forms of enzyme system get involved in their degradation. Such a situation will be in line with the economy of the cellular metabolism. The lower myoinositol phosphates i.e. myoinositol disphosphate and monophosphate in contrast are bad substrates for all the enzymes with the exception that of enzyme IE which acts on myoinositol diphosphate most preferably. As pointed above, the myoinositol monophosphate is the worst substrate for all the enzymes, nervertheless on comparative basis, enzyme IC and IB seem to hydrolyse it. Often the results based on K_m calculation do not reflect the correct affinity of the enzyme towards substrate. We have therefore analysed the data by using V_{max}/K_m values instead of K_m alone for determining the relative affinity of each enzyme towards various inositol phosphates. Results given in Table 10 clearly demonstrate that myoinositol hexaphosphate is the most suitable substrate for enzyme II followed by IE and IB. This observation is somewhat in line with K_m data except enzyme IB. Similarly myoinositol pentaphosphate is preferably hydrolysed by enzyme IE followed by enzyme II. The myoinositol tetraphosphate is a preferred substrate for both enzyme II and ID but the myoinositol triphosphate is the most suitable substrate for enzyme II. Diphosphates and monophosphates unlike the K_m data show preference for enzyme IE and II.

Considering the total results, one can predict to some extent the relative roles of various molecular species of phytase in the requlation of phytic acid metabolism and thereby in the regulation of mobilisation of stored phosphorous in germinating seedlings. Where as all enzymes seem to play important role, the enzyme IE and II appear to play a dominant role in the complete dephosphorylation of phytic acid as will be discussed in latter chapter. The level of these two enzymes increases continuously throughout the entire period whereas the other enzymes show a definite increase and decrease as a function of germination period. As far the substrate, specificity is concerned, there does not exist a clear cut preference, although significant differences in the specificity are clearly evident from the data. This information may be useful in determining the relative role of the various enzymes in phosphorus metabolism.

4.4 DISCUSSION

The comparison of some characteristic properties of various species of phytase as given in Table 8 clearly shows that the Cucurbita maxima phytase species can be divided into two types. The first type includes those which are relatively of higher molecular weight and separated by CM cellulose column chromatography. These molecular forms exhibit both similarity and dissimiliarity in properties. They also have significantly different molecular weights although the variation between different forms is small. As will be pointed out latter in the thesis, the de novo synthesis

of first type predominantly appears in the early period of germination. This type includes phytase IA, IB, IC and ID. The similiarity in their properties are in their Q_{10} values which lie at about 1.3 while K_m values, pH optima and thermal stability show very little variation. Among the marked differences in their properties, are large variation in V_{max} values, ΔH values, Ea, free energy change and ΔS values. The molecular weights and electrophoretic mobility also differ significantly.

The second type of phytases are those which have relatively low molecular weight, higher thermal stability, higher Q_{10} values (2.3) and low K_m values and relatively low value of ΔH, Ea and ΔS. This latter category of enzymes has two molecular species designated as phytase IE and phytase II. IE was separated along with the enzyme IA, IB, IC, ID by gel filtration and was first thought to be a high molecular species. But the values of molecular weights determined by SDS PAGE show that it is more closer to enzyme II and so is the case with some of the properties mentioned above. As will be shown in succeeding chapters, like phytase II, the de novo synthesis of phytase IE also increases through out the entire germination period.

On the basis of the substrate specificity there appears to be three types of phytases present in the germinating Cucurbita maxima cotyledons. The first type are preferentially concerned with the hydrolysis of phytic acid. Second type are those which show substrate preference for the

lower myoinositol phosphates (tetra and tri) while the third ones are those which do not have any specific preference but can act on all substrates. The relative concentrations of different inositol phosphates seem to control the activity level of various enzymes and thus controlling the overall phytate metabolism in germinating seedlings. This particular aspect will be dealt in more details in latter chapters.

The thermodynamic studies have been found to be very useful in characterizing the multimolecular forms of phytase. For instance, Ea, ΔG and ΔS values of phytase I are significantly different from those of phytase II. Since enzyme reactions are assumed to proceed by formation of an enzyme-substrate intermediate (164), and since breakdown of this complex is the slowest step, as indicated by Ea- the equivalent energy required to convert the enzyme-substrate complex into an activated molecule in accordance with the following equation:

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \rightleftharpoons ES^* \rightleftharpoons E + \text{products}$$

ES* = Activated complex.

From this consideration the enzyme II is a more efficient catalyst than enzyme I because under optimum assay conditions the Ea for phytase II is lower than that of I. This is further supported by the much lower value of entropy change (ΔS) of phytase II indicating that enzyme II undergoes a little conformational change during the formation of active enzyme-substrate complex.

Value of interaction constant of phytases with myo-inositol hexaphosphate was found to be nearly 1.0 indicating that the apparent number of myoinositol hexaphosphate molecules interacting per enzyme molecule was also one and the phenomenon of cooperativity was absent (162,163).

The positive values of ΔH for phytase catalysed reactions indicate that the hydrolysis of phytic acid by phytases is endothermic in nature. Values of ΔS and ΔG remain constant in the temperature range 30°-50°C showing that these 2 parameters of phytase hydrolysis reaction are not effected by change in temperature.

Q_{10} values for phytases between 30°-40°C were found to be ranging from 1.3 to 2.3. These results are in confirmity with the general trend of enzyme catalysed reaction which shows that the rate of reaction increases with rise in temperature (165). For the higher value of temperature coefficient, heat of activation of denaturation is higher(166).

The pH activity curves for each phytase species are sharply peaked giving a single optimum pH. This is indicative of only one ionic form of active site in different phytases which is most effective. Since the pH optima for phytases IA, IB, IC and ID vary within a narrow pH range (pH 4.8-5.2), the active sites of these enzymes may be closely resembling in regard to aminoacid makeup of the active site. In the case of enzyme IE and II, where the optimum pH vary significantly (4.4 and 5.6), the aminoacid makeup of the active site appears to be different. Thus phytase IE and II

appear to be distinct than other phytase species. This is also supported by other properties like relatively low molecular weight, higher electrophoretic mobility, higher Q_{10} and greater substrate affinity towards myoinositol hexaphosphate. This aspect however, has to be investigated further.

4.5 SUMMARY

1. The general and kinetic properties including pH and temperature effects characterizing the multiple forms of phytase from Cucurbita maxima (Phytase IA, IB, IC, ID, IE and II) were investigated using sodium phytate as substrate. The molecular weights of phytase IA, IB, IC, ID, IE and II, as determined by SDS PAGE, were found to be 62,256; 63,096; 43,657; 77,625; 38,905 and 29,512 Daltons, respectively. The low molecular weight enzymes phytase II and IE exhibit different properties than phytase IA, IB, IC and ID.

2. Entropy change, ΔS, free energy change ΔG and the value of 'n' for the enzyme substrate interaction were found to be independent of temperature in temperature range of 30°-$50^{\circ}C$.

3. All the species of phytase I are thermally stable upto $40^{\circ}C$ but phytase II is stable upto $50^{\circ}C$.

4. ΔH Values for all phytase forms are found to be positive indicating that enzyme catalyzed reactions are endothermic.

5. The different forms of phytase exhibit varying degree of substrate affinity towards myoinositol polyphosphate intermediates. It was found that phytase IA, IC, ID have maximum affinity for inositol tetraphosphate, but the enzyme IE and II show greater affinity for inositol diphosphate and inositol triphosphate, respectively. In addition, the phytase II exhibits high substrate affinity for all inositol polyphosphates which may be of physiological significance, as this enzyme makes its appearance at a very late stage of germination when both the level of phytase I activity and the concentration of phytate were very low.

TABLE-2: VALUES OF K_m AND V_{max} OF PHYTASES WITH PHYTIC ACID AS SUBSTRATE

Temp.	K_m ($mMX10^{-2}$) Phytase isoenzymes						V_{max} (mM/hr) Phytase isoenzymes					
°C	IA	IB	IC	ID	IE	II	IA	IB	IC	ID	IE	II
30	10.0	21.7	12.4	10.0	4.5	7.1	8.5	27.0	12.8	14.9	7.9	11.8
40	12.5	22.7	15.6	15.6	5.2	7.5	11.8	33.3	16.4	20.0	11.1	14.3
45	14.0	23.3	19.0	19.0	5.7	-	14.0	37.0	19.6	22.7	13.3	-
50	16.0	23.8	22.0	23.0	6.1	7.9	16.4	41.7	22.7	26.3	15.9	17.5

TABLE-3: ΔH AND Ea VALUES OF PHYTASES WITH PHYTIC ACID AS SUBSTRATE

Parameter	Phytase isoenzymes					
	IA	IB	IC	ID	IE	II
ΔH (Kcal/mole)	4,606	921	5,527	8,751	3,224	921
Ea (Kcal/mole)	4,606	5,527	5,989	6,448	6,909	3,685

TABLE-4: ΔG & ΔS VALUES OF PHYTASES WITH PHYTIC ACID AS SUBSTRATE

Temperature °C	Phytase isoenzymes IA	IB	IC	ID	IE	II
			ΔG (Kcal/mole)			
30	1,396	921	1,270	1,396	1,884	1,605
40	1,298	923	1,168	1,153	1,845	1,629
45	1,245	923	1,055	1,054	1,816	-
50	1,190	922	997	952	1,808	1,637
			ΔS (Kcal/mole/kelvin)			
30	10.590	0	14,050	24.300	4.400	2.260
40	10.570	-0.006	13.930	24.300	4.400	2.260
45	10.570	-0.006	14.060	24.200	4.400	-
50	10.580	-0.003	14.030	24.100	4.400	2.220

TABLE-5: Q_{10} VALUES OF PHYTASE ISOENZYMES

(The substrate used was myoinositol hexaphosphate)

Phytase isoenzymes	IA	IB	IC	ID	IE	II
*Q_{10} values	1.3	1.2	1.5	1.3	2.3	2.3

$$*Q_{10} = \frac{\text{Rate of reaction at } 40^{\circ}C}{\text{Rate of reaction at } 30^{\circ}C}$$

TABLE-6: ELECTROPHORETIC MOBILITIES OF PHYTASE ISO-ENZYMES IN POLYACRYLAMIDE GEL ELECTROPHORESIS

Enzyme	*Electrophoretic Mobility Ratio
Phytase IA	0.49
Phytase IB	0.48
Phytase IC	0.60
Phytase ID	0.50
Phytase IE	0.63
Phytase II	0.69

*Electrophoretic mobility ratio $= \frac{\text{Distance travelled by protein}}{\text{Distance travelled by tracking dye}} \times \frac{\ell'}{\ell}$

where,

ℓ = gel length before staining,
and ℓ' = gel length after staining

TABLE-7: ELECTROPHORETIC MOBILITIES OF STANDARD PROTEINS AND PHYTASE ISOENZYMES IN SDS-PAGE

Protein	Molecular wt. (Daltons)	Electrophoretic Mobility Ratio
	Standard Proteins	
1. Lysozyme	14,400	0.93
2. Soyabean trypsin inhibitor	21,500	0.79
3. Carbonic anhydrase	31,000	0.71
4. Ovalbumin	45,000	0.61
5. BSA	66,200	0.50
6. Phosphorylase B	92,500	0.39
7. β Galactosidase	116,250	0.31
8. Myosin	200,000	0.03
	Enzymes	
9. Phytase IA	-	0.50
10. Phytase IB	-	0.49
11. Phytase IC	-	0.60
12. Phytase ID	-	0.43
13. Phytase IE	-	0.63
14. Phytase II	-	0.71

TABLE-8: SOME CHARACTERISTICS OF PHYTASE ISOENZYMES

(All the parameters were measured using myoinositol-hexaphosphate as substrate and at 40°

Kinetic Parameters	Phytase isoenzymes					
	IA	IB	IC	ID	IE	II
Optimum pH	5.0	4.8	5.0	5.2	4.4	5.6
Thermal Stability	40°C	40°C	40°C	40°C	40°C	50°C
$Q_{10}(Q_{40-30})$	1.3	1.2	1.5	1.3	2.3	2.3
K_m values($mM.10^{-2}$)	12.5	22.7	15.6	15.6	5.2	7.5
V_{max} values(mM/hr)	11.8	33.3	16.4	20.0	11.1	14.3
ΔH(Kcal/mole)	4606	921	5527	8751	3224	921
Ea(Kcal/mole)	4606	5527	5989	6448	6909	3685
ΔG(Kcal/mole)	1298	923	1168	1153	1845	1629
ΔS(Kcal/mole/Kelvin)	10.57	-0.006	13.930	24.300	4.400	2.260
Molecular wt.(Daltons)	60,256	63,096	43,657	77,625	38,905	29,512
Electrophoretic Mobility Ratio	0.49	0.48	0.60	0.50	0.63	0.69

TABLE-9: SUBSTRATE SPECIFICITY OF PHYTASE FORMS TOWARDS VARIOUS MYOINOSITOL PHOSPHATE ESTERS

Substrate	Phytase isoenzymes					
	IA	IB	IC	ID	IE	II
	K_m values (mM x 10^{-2})					
Myoinositol monophosphate	25.0	20.0	18.2	50.0	33.0	25.0
Myoinositol diphosphate	13.9	17.0	11.9	11.1	6.0	10.0
Myoinositol triphosphate	6.4	6.8	7.4	7.0	14.3	5.6
Myoinositol tetraphosphate	6.0	5.9	6.8	5.9	13.3	6.2
Myoinositol pentaphosphate	9.6	7.5	8.0	7.0	14.3	6.5
Myoinositol hexaphosphate	12.5	22.7	15.6	15.6	5.2	7.5
	V_{max} values (mM/hr)					
Myoinositol monophosphate	11.1	9.1	7.0	25.0	40.0	20.0
Myoinositol diphosphate	7.7	10.0	6.3	7.4	7.1	10.0
Myoinositol triphosphate	3.5	10.0	10.0	10.0	14.3	12.5
Myoinositol tetraphosphate	3.0	7.1	10.0	16.7	14.3	16.7
Myoinositol pentaphosphate	5.9	10.0	7.7	8.7	33.3	11.1
Myoinositol hexaphosphate	11.8	33.3	16.4	20.0	11.1	14.3

TABLE-10: V_{max}/K_m VALUES OF PHYTASE ACTION ON VARIOUS MYOINOSITOL PHOSPHATE ESTERS

Substrate	V_{max}/K_m (hr^{-1}) for Phytase isoenzymes					
	IA	IB	IC	ID	IE	II
Myoinositol hexaphosphate	94	147	105	128	214	191
Myoinositol pentaphosphate	62	133	96	124	233	171
Myoinositol tetraphosphate	50	120	147	283	108	269
Myoinositol triphosphate	55	147	135	143	100	223
Myoinositol diphsophate	55	59	53	67	118	100
Myoinositol monophosphate	44	46	39	50	121	80

Fig. 10 : EFFECT OF pH ON DIFFERENT FORMS OF CUCURBITA MAXIMA PHYTASE.

Each of the phytase enzyme was incubated with sodium phytate (0.25 mM) for 1 hr at 40°C. The buffer solutions were adjusted to the desired pH with the help of sodium acetate and acetic acid/NaOH solutions, before adding it to the reaction mixture. The reaction mixture and assay conditions were those as described in the methods. Results are plotted as percent of maximum as a function of pH. -○○- IA, -●●- IB, ⊖⊖ IC, -△△- ID, -▲▲- IE, -□-□- II.

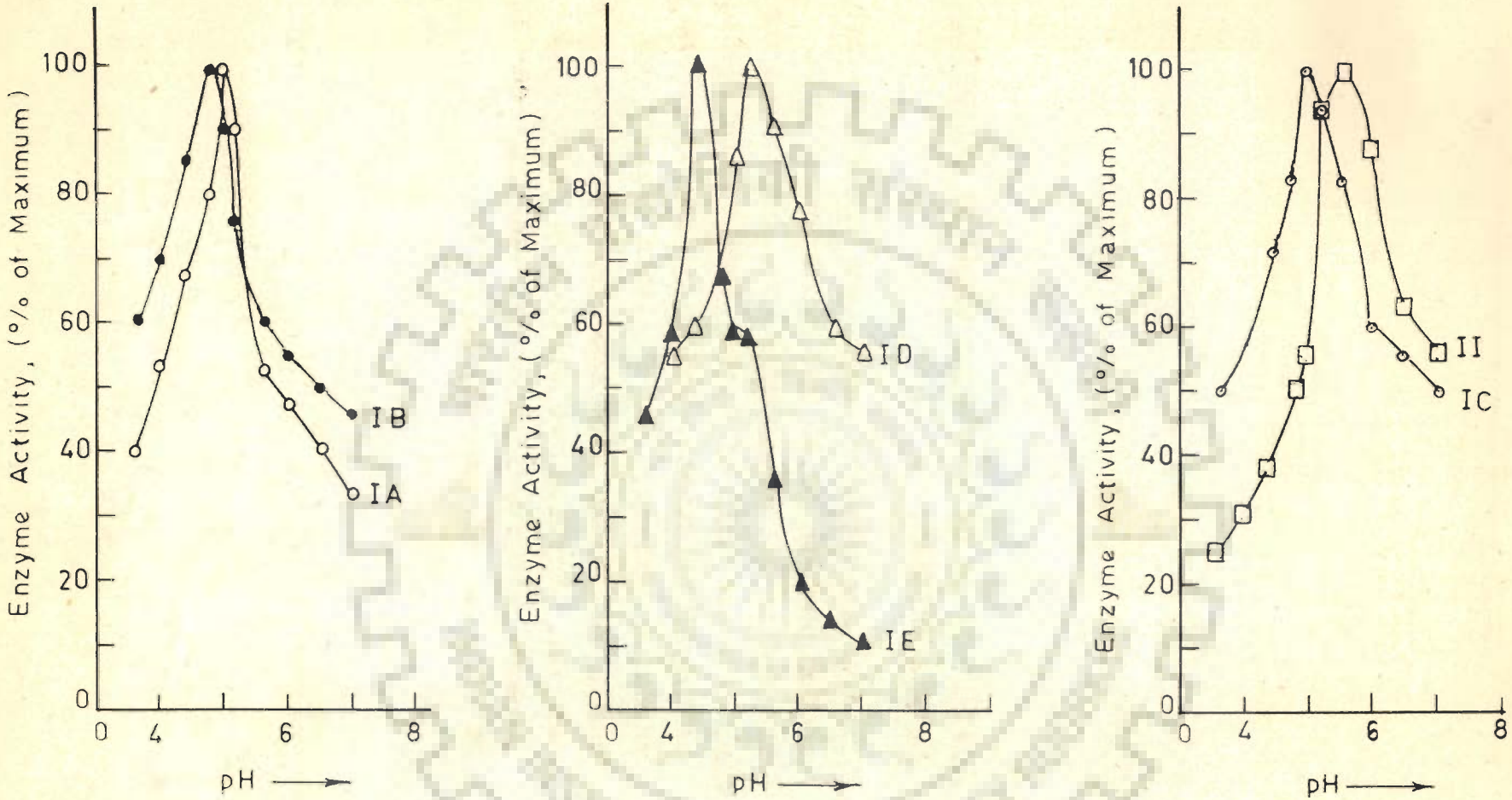


Fig. 10 - pH Optima Curves

Fig. 11 : THERMAL STABILITY CURVES OF PHYTASE ISOENZYMES.

The enzymes were first exposed to different temperatures (20°-80°C) at optimum pH for 60 min., allowed to cool in the bath for 60 min and then assayed for the residual activity by standard procedure described in Chapter III. The controls (without heat treatment) were run simultaneously and used as 100 %. The percent residual activity was plotted versus temperature -ΘΘ- IA, -●●- IB, -⊙-⊙- C, -△-△- ID, -▲-▲- IE, -□□- II.

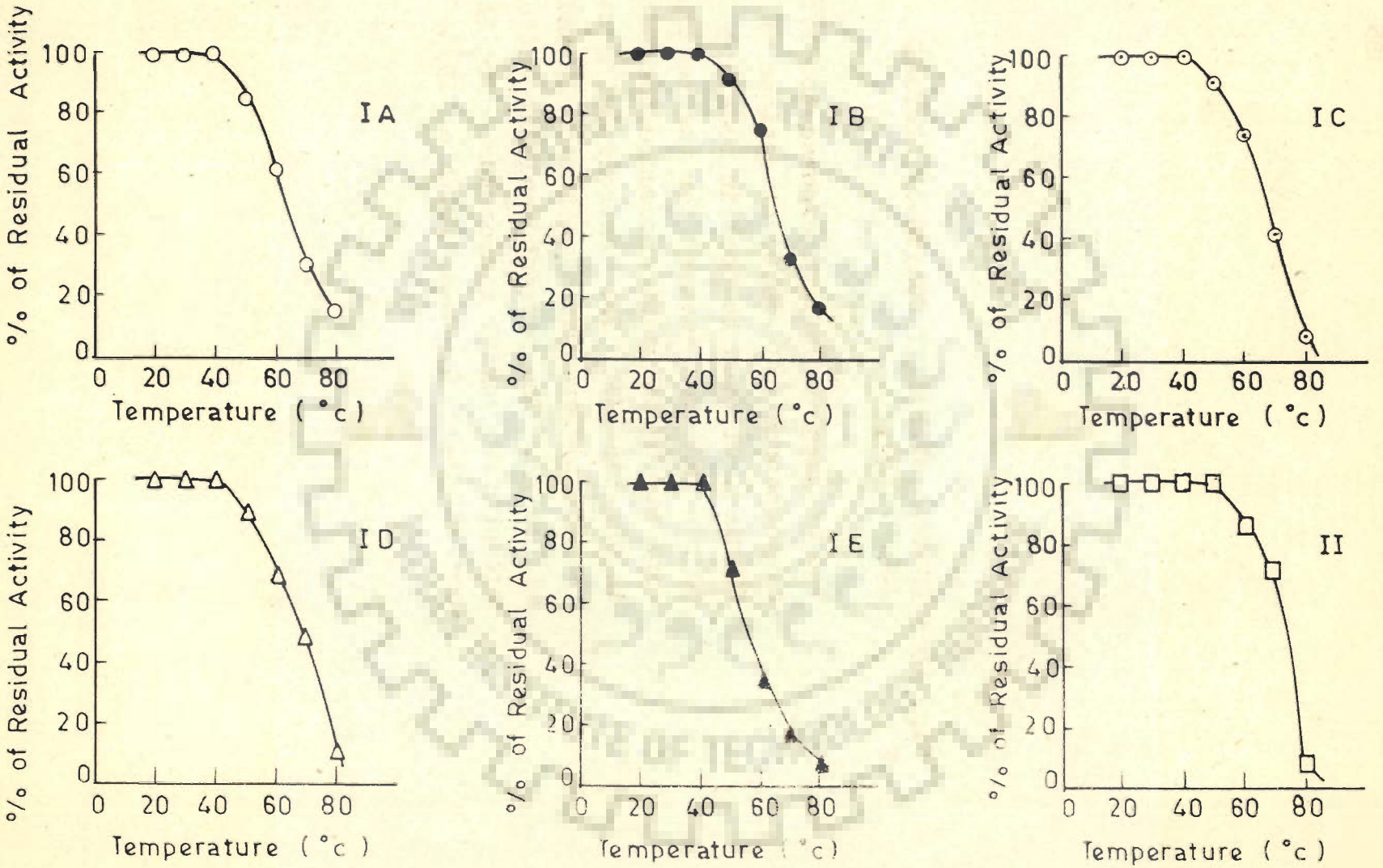


Fig. 11 - Thermal Stability Curves

Fig. 12 : LINEWEAVER BURK PLOTS OF PHYTASE ISOENZYMES.

The data for the Lineweaver Burk plots ($^{1}/_{V}$ vs 1/(S)) were obtained by measuring the activity of various phytase forms using varying substrate (phytate) concentration (0.01 mM -2.00 mM) at optimum pH under standard assay conditions described in Chapter III. -O-O- IA, -●●- IB, -⊙-⊙- IC, -△-△- ID, -▲▲- IE, -□□- II.

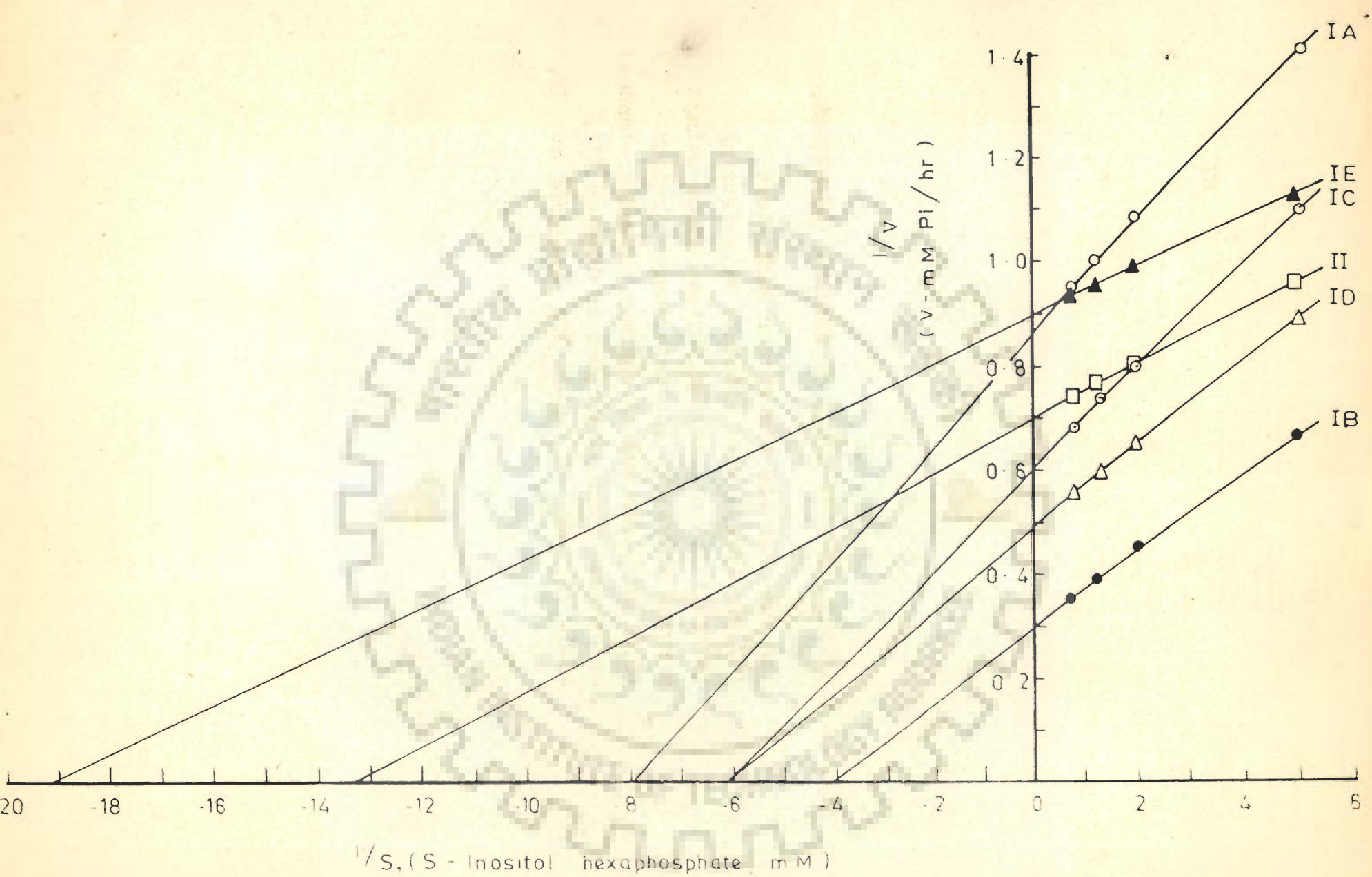


Fig 12 Lineweaver Burk Plots of Phytases with Inositol hexaphosphate

Fig. 13 : PLOTS OF LOG K_m versus 1/T FOR PHYTASE ISOENZYMES.

K_m values at different temperatures for different phytase enzymes were determined from Lineweaver Burk plots. From the slopes of linear curves of different isoenzymes between $\log_{10} K_m$ and 1/T , value of ΔG was determined (Section 4.2.2.2.3.3). -O-O- IA, -●●- IB, -◐-◐- IC, -△-△- ID, -▲-▲- IE, -□□- II.

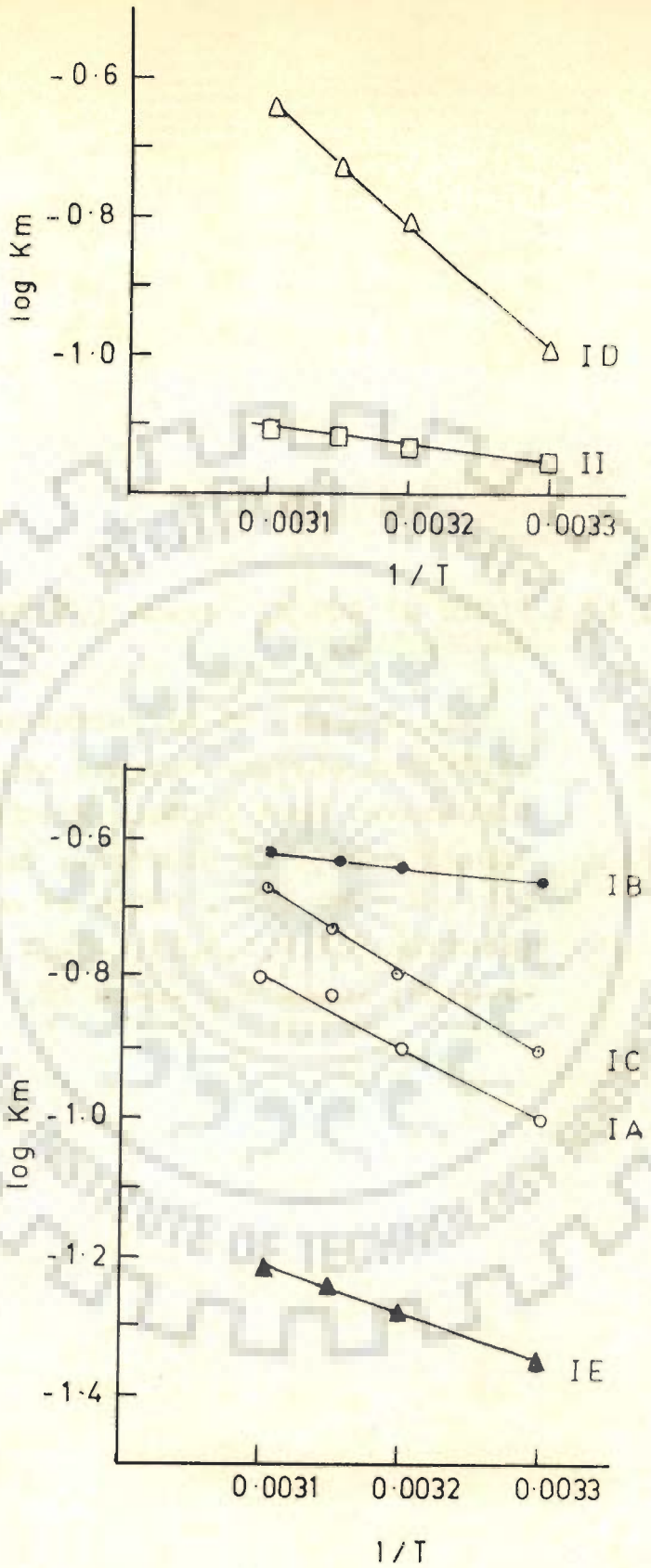


Fig. 13 - Plots of Log Km vs 1/T

Fig. 14 : HILL PLOTS OF PHYTASE ISOENZYMES

Hill plots of various phytase species were obtained by plotting the values of log $(V/V_{max}-V)$ versus log (S), where V_{max}, V and (S) represent maximum velocity, initial velocity and the substrate concentration respectively. The interaction constant, i.e. the number of substrate molecules interacting per enzyme molecule, was determined from the slope of each plot. -O-O- IA, -●●- IB, -⊙-⊙- IC, -△-△- ID, -▲▲- IE, -▣▣- II.

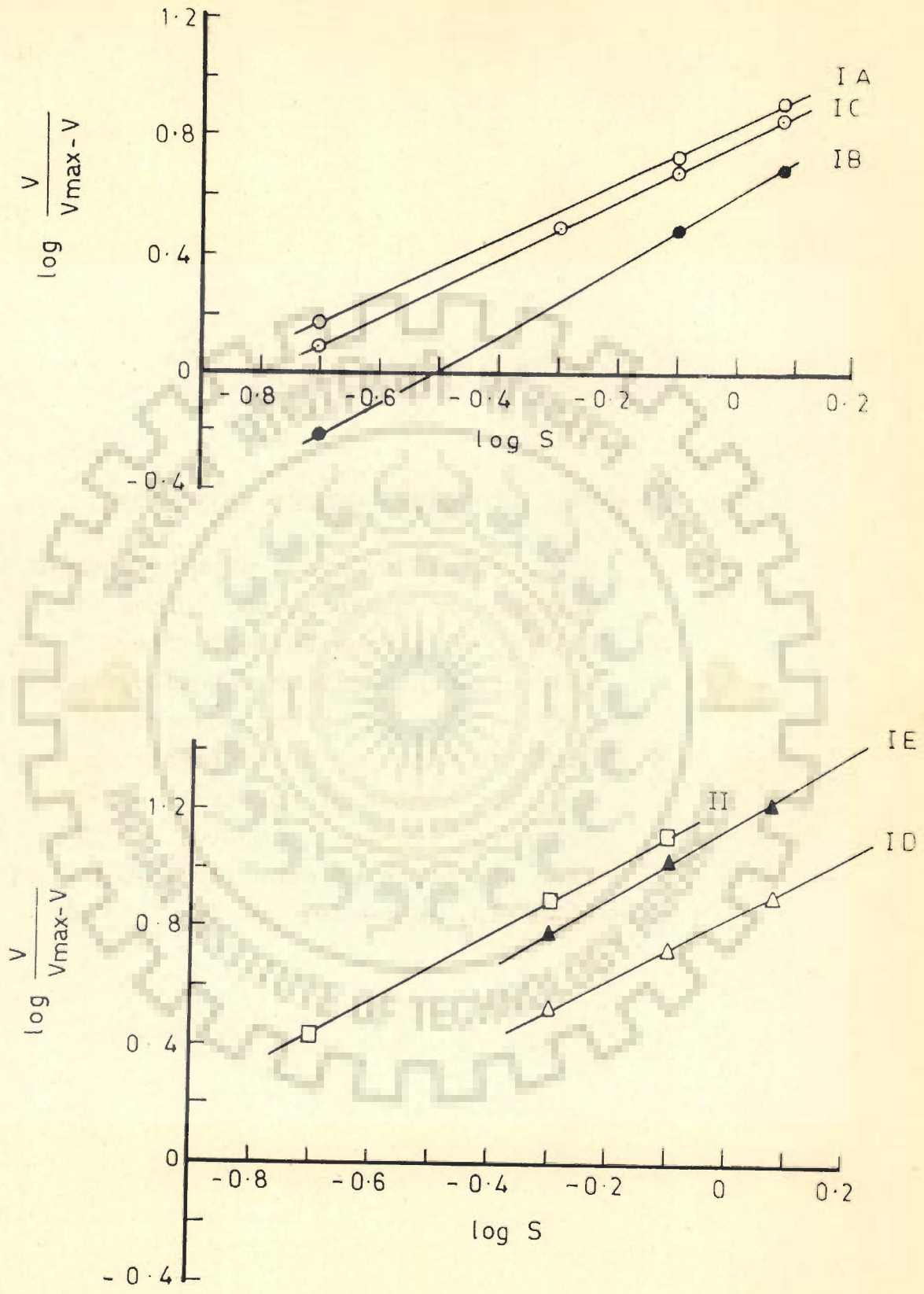


Fig. 14 - Hill's Plots

Fig. 15 : ACTIVATION ENERGY OF PHYTASE ISOENZYMES.

The energy of activation, Ea, for various phytase forms was obtained from the slope of the plots of log V_{max} versus 1/T. The V_{max} values (in mM/hr) for each enzyme were obtained by the double reciprocal plots of 1/V versus 1/(S) under optimum assay conditions. -O-O- IA, -●-● IB, -◉-◉- IC, -△-△- ID, -▲-▲ IE, -□-□- II.

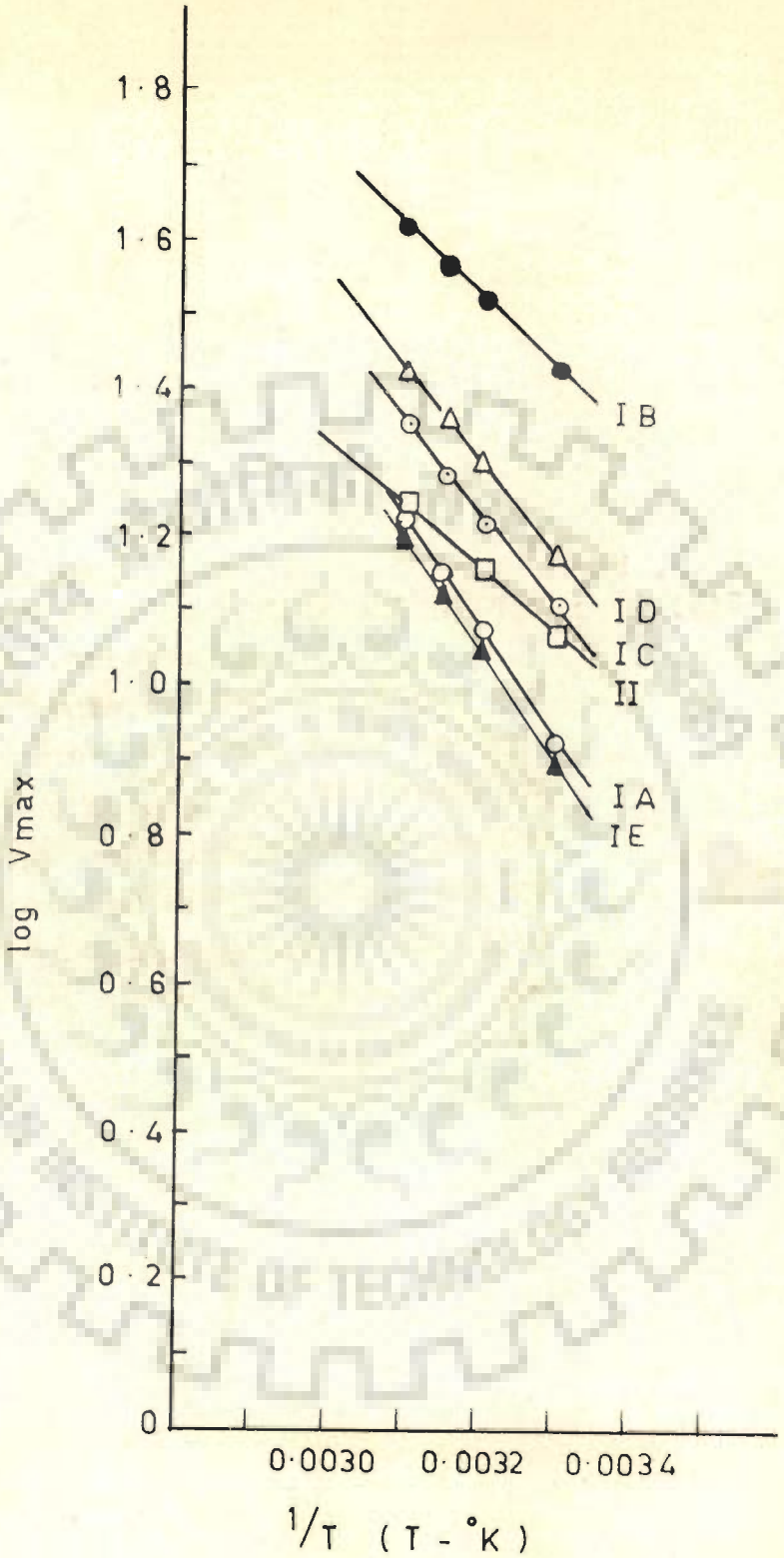


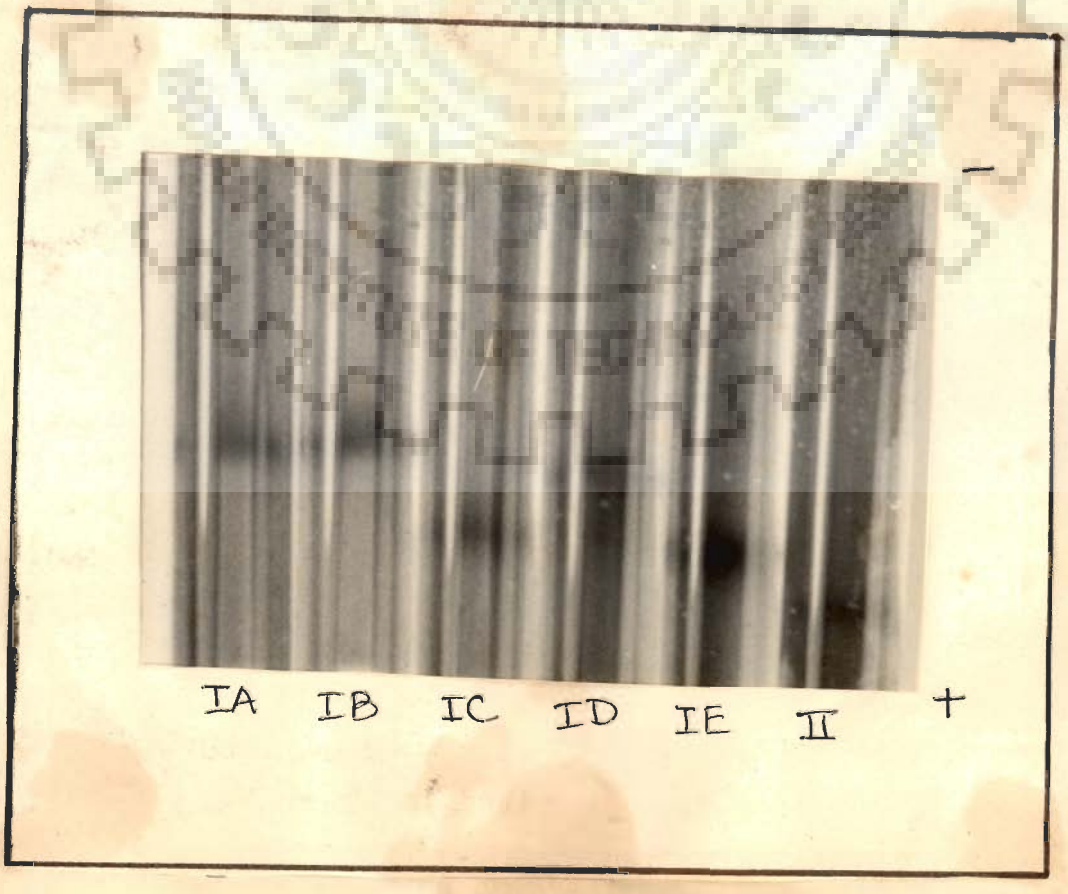
Fig. 15 - Arrhenius Plots

Fig. 16 : ELECTROPHORETIC PATTERNS OF PHYTASES IN SDS PAGE AND POLYACRYLAMIDE GEL ELECTROPHORESIS.

The SDS PAGE electrophoresis of various forms of phytases was carried out on 12.6 % polyacrylamide gel columns in 0.1M phosphate buffer, pH 8.3, containing 0.1 % SDS, using bromophenol blue as the tracker dye. A current of 5 mA per tube was passed constantly for 150 min. The electrophoresis of a standard mixture of proteins (containing lysozyme, soyabean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, β galactosidase, myosin) of known molecular weights was also carried out along with phytases. The protein bands were located by amidoblack. The details of procedure are given in text.

In case of polyacrylamide gel electrophoresis, the electrophoresis of phytases was carried out on 7.5 % polyacrylamide gel columns in 0.1M phosphate buffer pH 7.2, Bromophenol blue was used as tracking dye. A current of 5mA per tube was passed continuously for 120 min. The protein bands were stained by amidoblack. The method used is described in the text.

SDS - PAGE



PAGE

Fig. 17 : CALIBRATION CURVE OF STANDARD PROTEINS FOR DETERMINATION OF MOLECULAR WEIGHTS OF MULTI-MOLECULAR FORMS OF PHYTASE.

Electrophoresis of the following protein standards was done alongwith enzyme samples by SDS-PAGE method : soyabean trypsin inhibitor (MW 21,500 Daltons), carbonic anhydrase (MW 31,000 D), Ovalbumin (MW 45,000 D), BSA (MW 66,200 D), phosphorylase B (MW 92,500 D), β-galactosidase (MW 116,250 D), lysozyme (MW 14,400 D), and myosin (MW 200,000 D). The calibration curve was obtained by plotting log molecular weight versus electrophoretic mobility of standard proteins. Other details of the procedure are given in the text.

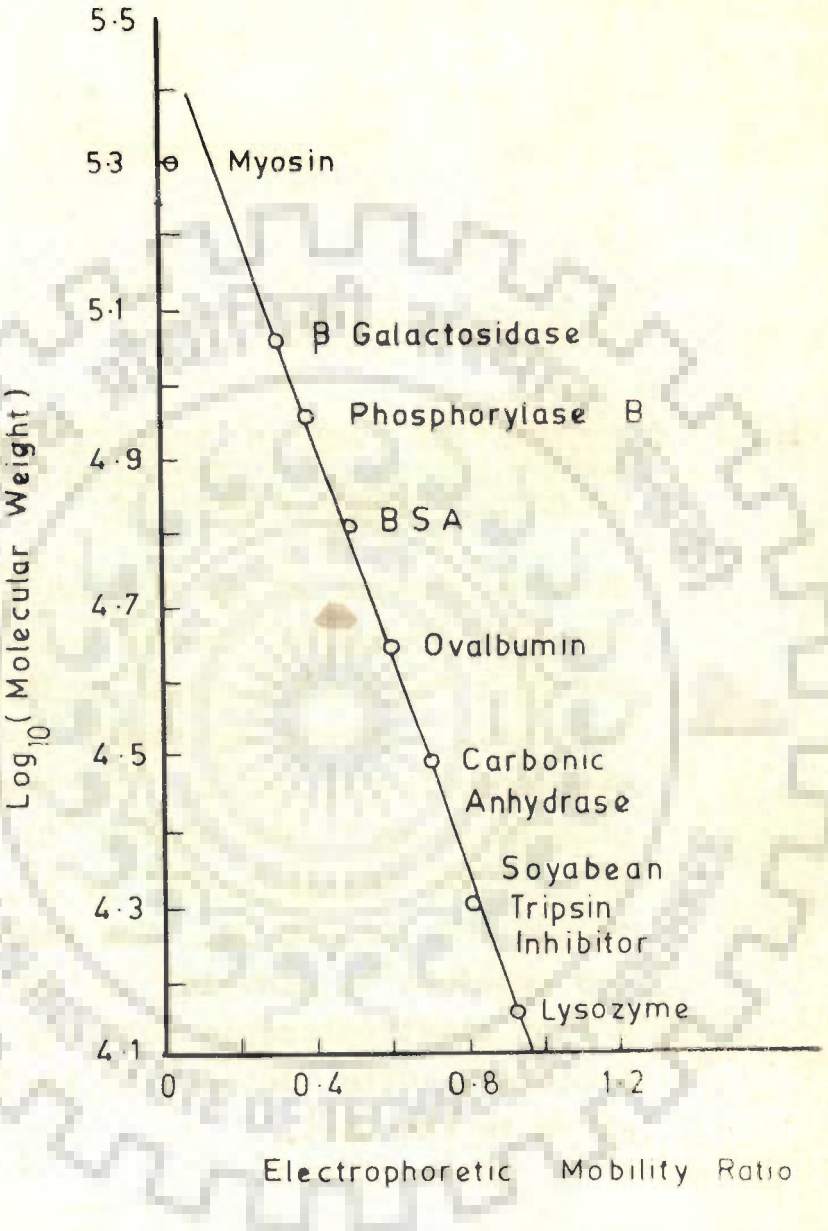


FIG. 17 - Electrophoretic Mobilities of Standard Proteins in SDS PAGE

Fig. 18 : LINEWEAVER BURK PLOTS OF PHYTASE ISOENZYMES' ACTIVITY ON DIFFERENT MYOINOSITOL PHOSPHATE ESTERS.

Equal amounts of various phytase isoenzymes were incubated with varying concentrations of myoinositol phosphate esters (0.02 -2.00 mM) in 0.1M acetate buffer of optimum pH at 40°C for 60 min. Rate of reaction was measured as mM phosphate released per hour. The double reciprocals 1/v versus 1/(S) were plotted and the values of K_m and V_{max} for the substrate used were determined from the intercepts on X axis and Y axis, respectively. -O-O- IA, -●●- IB, -⊙-⊙- IC, -△-△- ID, -▲-▲- IE, -▫-▫- II.

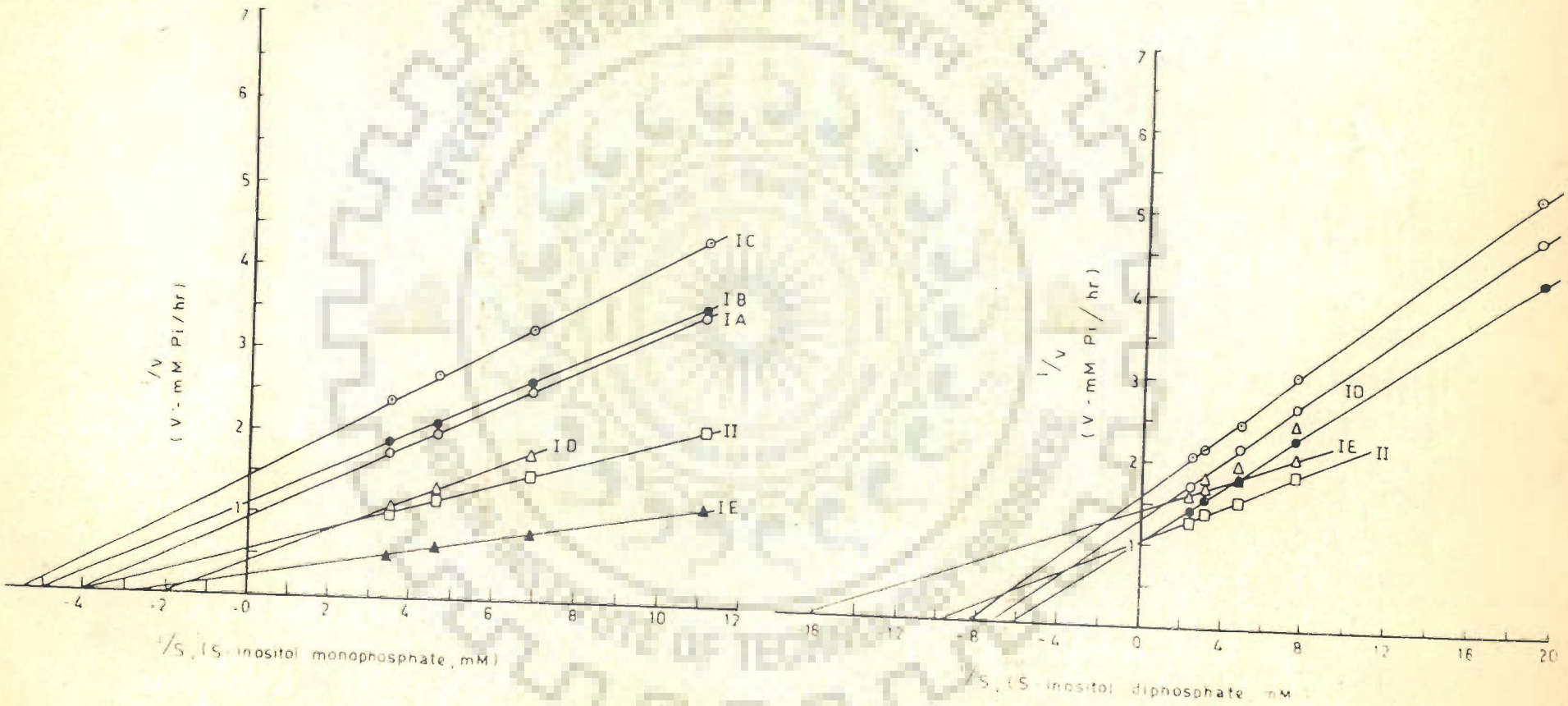


Fig 18.1 - Lineweaver Burk Plots of Phytases with Different Inositol Phosphate Esters

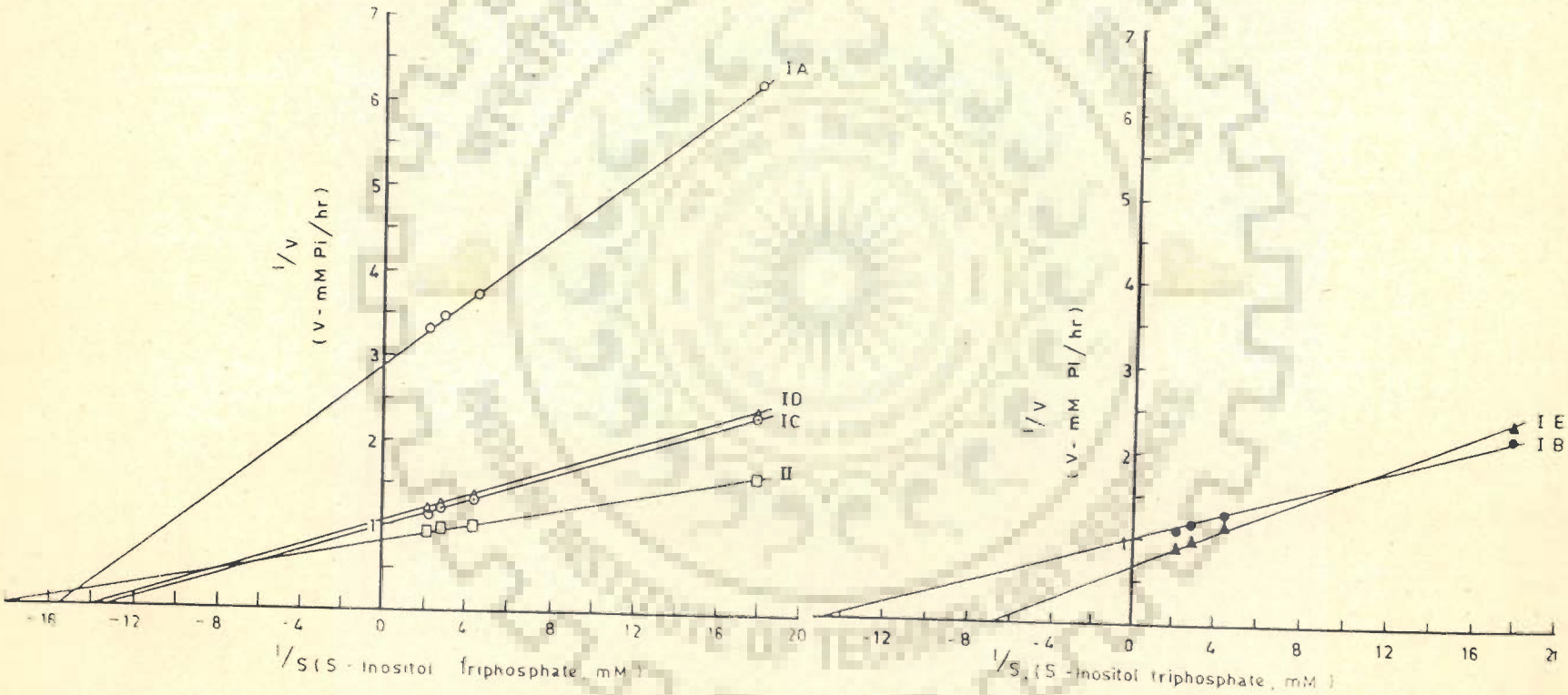
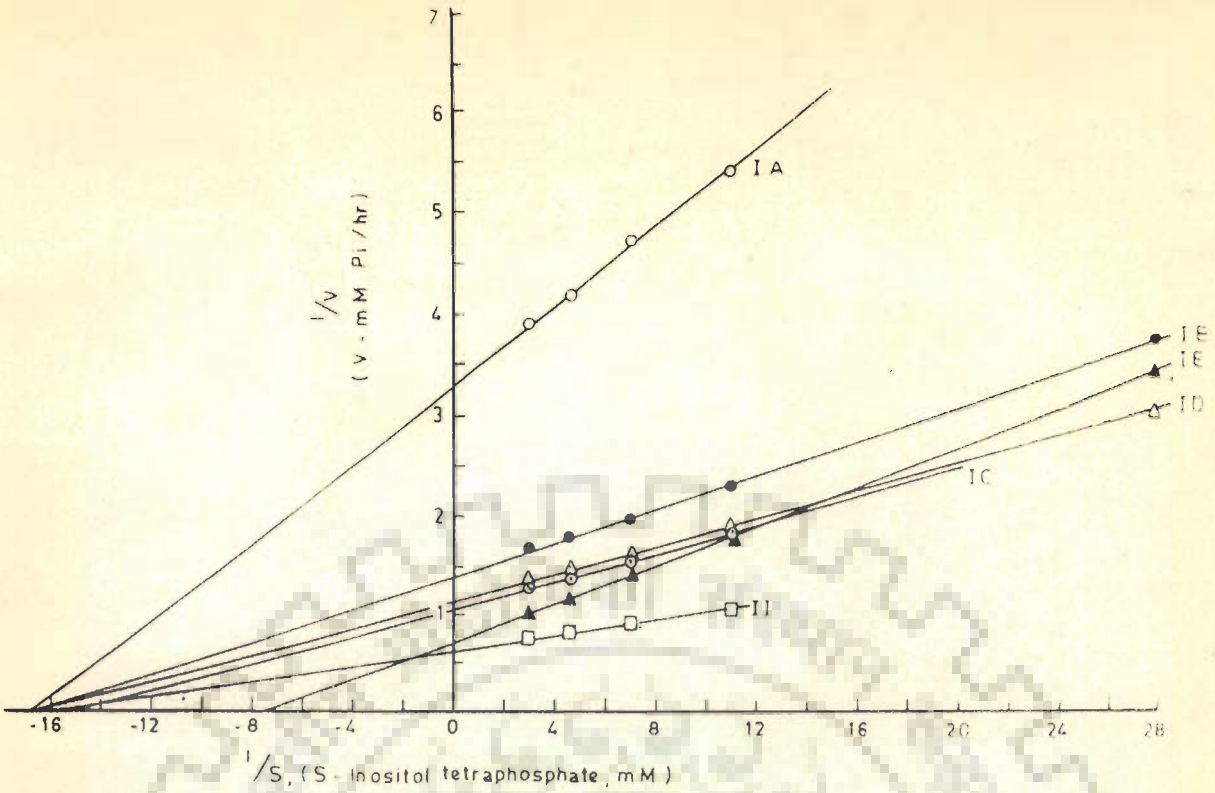


Fig 18.2 . Lineweaver Burk Plots of Phytases with Different Inositol Phosphate Esters



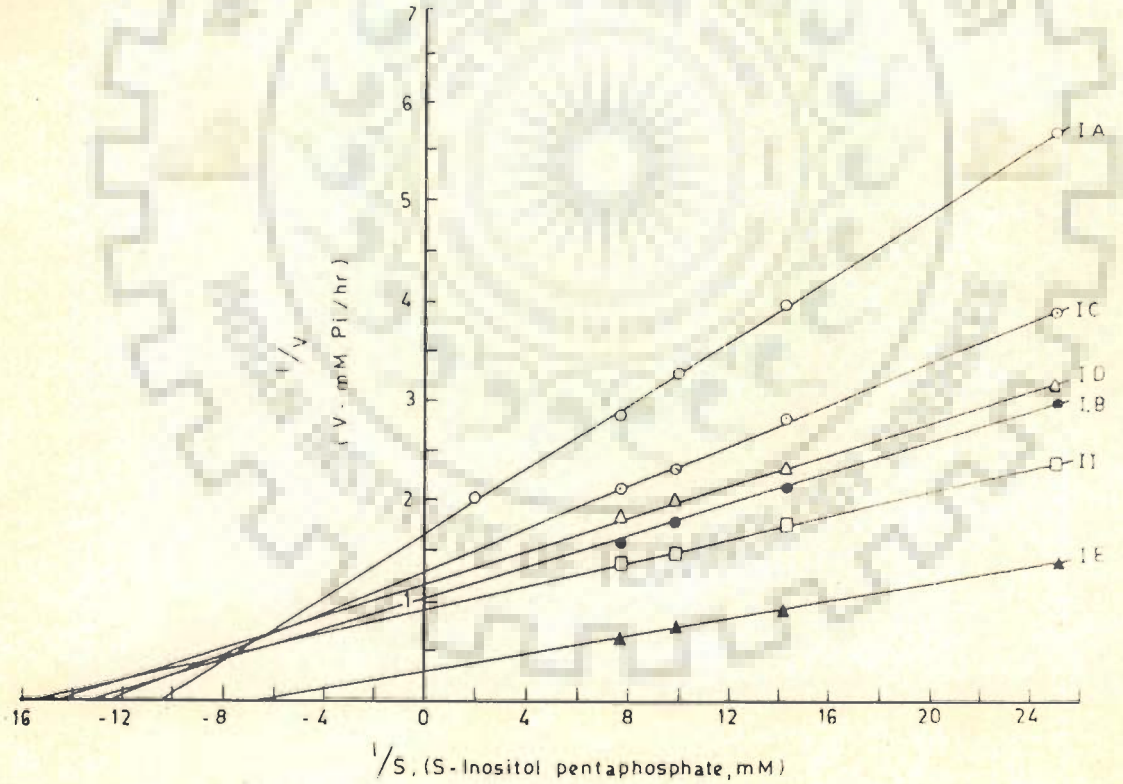


Fig. 18.3 - Lineweaver Burk Plots of Phytases with Different Inositol Phosphate Esters

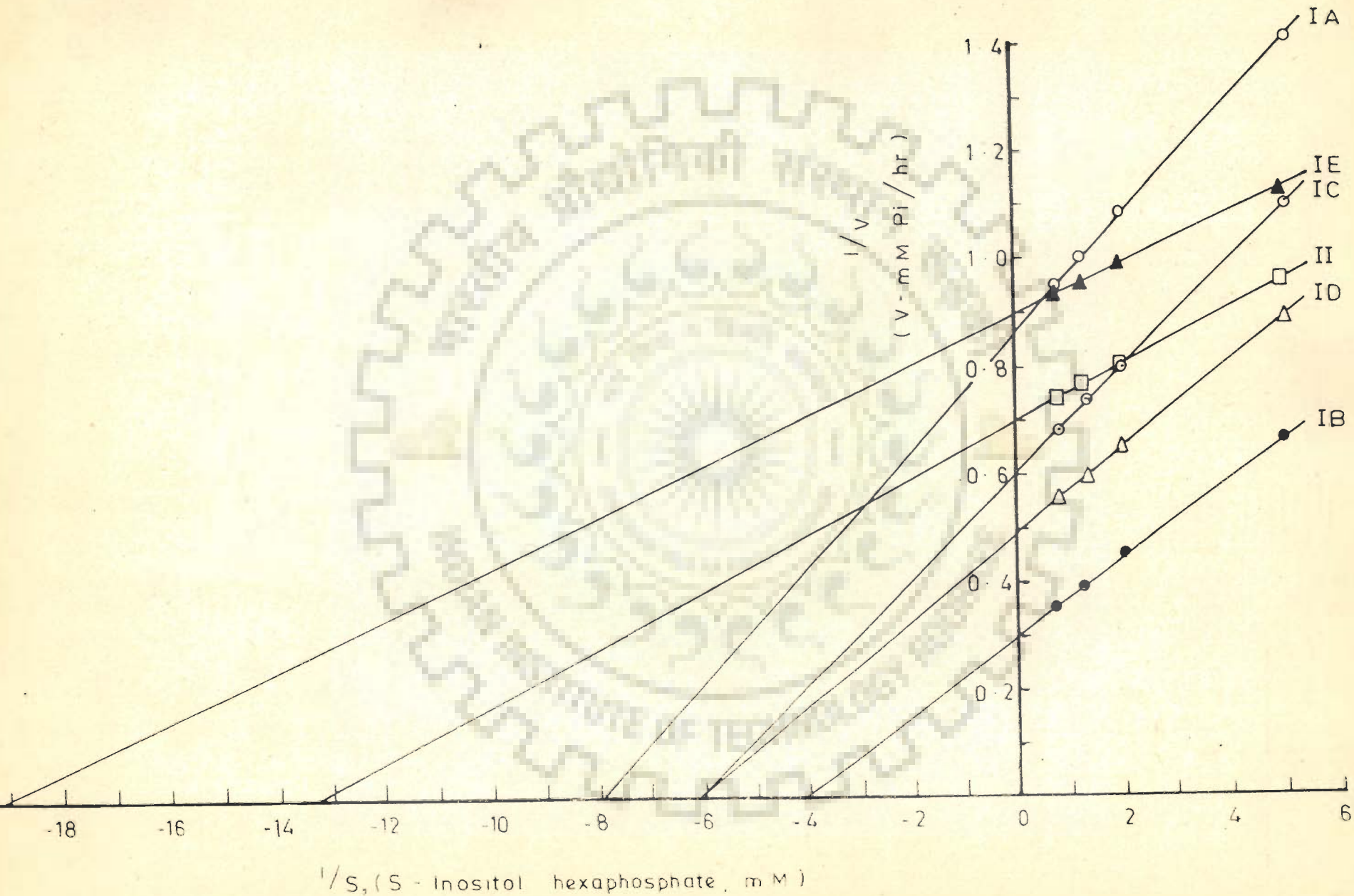


Fig 18.4 Lineweaver Burk Plots of Phytases with Different Inositol Phosphate Esters

CHAPTER V

DEPHOSPHORYLATION PATHWAY OF MYOINOSITOL HEXAPHOSPHATE BY DIFFERENT PURIFIED SPECIES OF PHYTASE IN VITRO

5.1 INTRODUCTION

In previous chapter the properties of various phytase species were reported. The kinetic data showed that in isolated systems, each of the phytase forms, was capable of dephosphorylating phytic acid, though the degree of efficiency varied from one form to another. Since the enzymatic dephosphorylation of phytic acid is known to proceed in a stepwise manner, it is likely that the different phytase forms also function in the same way. However, whether all of these follow the same pathway, or is there a different pathway for each isoenzyme was not clear from the kinetic parameters.

In this chapter we report the results of phytic acid dephosphorylation pathway followed by different purified phytase species in an isolated system. The approach has been to bring about the partial hydrolysis of phytic acid as to the various intermediates followed by their isolation, purification and characterization.

5.2 MATERIALS AND METHODS

5.2.1 Materials

The purified forms of phytase isoenzymes used in the dephosphorylation study were prepared from the Cucurbita maxima seeds as described in Chapter III.

Dowex AG 50W-X8 (50 mesh), H^+ form, and Dowex AG 1-X8

(400 mesh), Cl^- form, were procurred from Bio Rad Laboratories, Richmond, California. D-Sorbitol, D-erythrose, D-altrose, D(-)ribose were obtained from Sigma, St.Louis, USA and L(+) arabinose from BDH, Poole, England. All other chemicals used were of analytical grade.

5.2.2 Methods

5.2.2.1 Partial Dephosphorylation of Phytic Acid by Purified Forms of Pumpkin Phytase Isoenzymes

Partial dephosphorylation of phytic acid by various phytase isoenzymes was carried out by the following procedure. Sufficient amount of purified phytase isoenzyme was added to 100 ml of 2mM phytate solution in 0.1M sodium acetate buffer of optimum pH and incubated at 40°C for 48 hr. After the completion of the reaction, the enzyme action was terminated by placing the reaction mixture in the boiling water bath for 15 min. The mixture was allowed to cool in ice and then extracted with equal volume of 20% trichloroacetic acid for half an hour. The solution was centrifuged at 12,000 Xg for 30 min to remove the denatured proteins. To the clear supernatant containing mixture of myoinositol phosphates, an excess of barium acetate was added (174) and the pH of this supernatant was adjusted to 8 with 10 NaOH. At this pH barium salts of myoinositol phosphates except the myoinositol monophosphate (MIP) were precipitated which were separated by centrifugation. From the supernatant MIP_1 was precipitated as lead salt by the addition of lead acetate (68). Barium salts of all the myoinositol phosphates were decationized by Dowex-50X8 (H^+ form) to obtain myoinositol phosphates in free acid forms in solution.

The lead salts was converted into free acid by H_2S in acidic solution.

5.2.2.2. Ion Exchange Chromatography of Myoinositol Phosphates on Dowex-1X8 (Cl^-)

Myoinositol phosphates were further saparated on a Dowex-1X8 (Cl^-) column (2 x 30 cm) by linear gradient elution with 0 to 1 M NaCl(175). 250 ml of 1 M NaCl solution and 250 ml of distilled water were filled in the reservoir and the mixing chamber of gradient system respectively. The rate of elution was 0.5 ml/min. 5 ml fractions were collected and analysed for total phosphorus by the Bartlett method (176). Tubes representing a single peak were pooled and the barium salts of myoinositol phosphates were precipitated from the pooled fractions and separated by centrifugation as before. The precipitate was suspended in distilled water and ionized with Dowex-50X8.

5.2.2.3 Identification of Myoinositol Phosphates

5.2.2.3.1 Determination of Phosphorus to Myoinositol ratio in Myoinositol Phosphates

Samples of myoinositol phosphates obtained from Dowex-1X8 (Cl^-) chromatography were hydrolyzed in 6 N HCl in sealed tubes for 72 hr in oven at 120°C. After cooling the tubes were opened and the contents were evaporated in vacuum. The last traces of HCl were removed by drying over NaOH pellets in a vacuum desiccator. The residue left was dissolved in 20 ml of distilled water. The total phosphate and myoinositol in hydrolyzate were determined by Fiske and Subba Row's periodate oxidation method (173), respectively. In the latter method

5 ml solution of 0.02M sodium metaperiodate was added to each sample (10 ml) including the standard solutions of inositol (5-40 μm) and incubated at 50°C for $2\frac{1}{2}$ hr. After the incubation period was over, the mixture was cooled and 2 ml of 12% solution of Na_2HPO_4 in 0.2 N H_2SO_4 and 5 ml of 10% KI (w/v) (in half saturated NaCl solution) were added to the flask. The liberated iodine was measured by titration with 0.001M $Na_2S_2O_3$ solution. A blank solution without inositol was also titrated. Myoinositol concentrations in each test sample were measured with the help of a calibration curve made by using myoinositol standards concurrently. Thus the ratios of P_i to myoinositol in the phosphate esters of myoinositol separated either by Dowex 1X8 (Cl^-) column chromatography or by paper chromatography were determined.

5.2.2.3.2 Paper Chromatography of Myoinositol Phosphates.

Various pyosphate esters of myoinositol isolated by Dowex-1X8 (Cl^-) column were further resolved and purified by the ascending paper chromatography on Whatman No.1 filter paper using 2-propanol-ammonia-water (5:4:1) as solvent system at 30°C (91). The samples of myoinositol phosphates were loaded on the paper and fully developed in preequilibrated chromatographic chamber with the above solvent for 36 hr. The paper chromatograph was dried in air, sprayed with Axelrod and Bandurski reagent(177) (prepared by mixing 45 ml of 70% $HClO_4$ (w/v), 10 ml conc. HCl, 1 g EDTA sodium salt and 945 ml of 1% ammonium molybdate). It was heated for 2 min at 85°C in a hot air oven and then exposed to UV light for

20 min. The blue spots were formed locating the presence of inositol phosphates. In each case phytic acid was used as reference.

5.2.2.3.3 Periodate Oxidation-Reduction of Inositol Polyphosphates to Corresponding Sugar Alcohols

The periodate oxidation-reduction was performed according to the procedure of Lim and Tate(75). The isolated myo-inositol di-,tri- and tetraphosphates (5-50 mg) were dissolved separately in 20 ml of 0.04 M sodium metaperiodate, pH 6.5. The reaction mixture was incubated at 25°C for 30 hr, time required for complete oxidation of unphosphorylated vicinal hydroxyl group of myoinositol. The contents of the reaction were cooled to 0°C in ice bath. 10 ml of 5% (w/v) chilled aqueous solution of $NaBH_4$ was slowly added to this reaction mixture and allowed to stand overnight at room temperature. After reduction was complete, the pH of the reaction mixture was adjusted to 4.8 by acetic acid and the sample was dephosphorylated by heating the reaction mixture at 120°C for 7 hr in sealed tubes. After complete dephosphorylation, the contents of tube were treated batch wise with Dowex-50 (H^+) resin to remove metal ions. The resin was filtered and the filtrate was evaporated to dryness. The dried residue was dissolved in methanol and evaporated to dryness. This step was repeated several times to ensure the complete removal of boric acid. The sugar alcohols thus formed from inositol polyphosphates were used for further analysis.

5.2.2.3.4 Acid Catalysed Phosphate Migration Across Cis hydroxyls

The migration of orthophosphate groups to the adjacent cis-OH group was attained by heating the aqueous solutions of myoinositol di-,tri- and tetra phosphates at 100°C in a boiling water bath. An equal volume of hot 2N HCl was then added and the solution was further heated at 100°C for 10 minutes, followed by neutralization by the addition of 1 N NaOH and cooling. The periodate oxidation procedure described above was employed and the sugar alcohols obtained were compared by paper chromatography. The sugar alcohols were dried under vacuum for IR spectroscopy.

5.2.2.3.5 Infra red Spectrometry of Sugar Alcohols

Further characterization of sugar alcohols obtained from the periodate oxidation procedure was achieved by IR spectrometry. Standard sugars were reduced to alcohols by $NaBH_4$ treatment as described earlier and used as reference standards for identification of sugar alcohols.

IR Spectra were scanned using the KBr disc method. 0.05 to 0.10mg of each sample was ground thoroughly with 400 mg of infra red grade KBr with an agate mortar and pestle. The samples were dried over P_2O_5 in vacuum desiccator. KBr discs were prepared with a Beckman dye at 20,000 pounds total pressure and spectra were measured in a double beam Beckman IR-20 spectrometer against a blank KBr disc.

5.2.2.3.6 Identification of Myoinositol Pentaphosphate

In order to determine the structure of myoinositol

pentaphosphate following experiments were carried out.

5.2.2.3.6.1 High Voltage Paper Electrophoresis

The high voltage paper electrophoresis of myoinositol pentaphosphate was carried out on Whatman No.1 chromatography paper in 0.1M oxalate buffer, pH 1.5, at 80 volts/cm for 30 min. as described by Seiffert and Agronoff(178). Phosphorus bands on dried electrophoretogram were identified by the method of Rorem(179).

5.2.2.3.6.2 Optical Rotation Measurement

Molar optical rotation of myoinositol pentaphosphate was measured by visual polarimeter using sodium D-line as light source. The temperature was kept 25°C.

5.2.2.3.6.3 Nuclear Magnetic Resonance

Phosphorus-31 nuclear magnetic resonance of myoinositol pentaphosphates were obtained with JEOL JNM FX100 FT Multinuclei NMR spectrometer operated at 40.27 MHz. Concentrated solutions of samples of myoinositol pentaphosphates were prepared in distilled water. 85% H_3PO_4 was used as external reference.

5.3 RESULTS AND DISCUSSION

5.3.1 Formation and Isolation of Myoinositol Phosphate Intermediates During the Hydrolysis of Phytic Acid by Different forms of Phytase

The myoinositol phosphate intermediates formed during the 48 hr hydrolysis of phytic acid by the individual phytase isoenzymes of Cucurbita seeds were separated by ion-exchange chromatography on Dowex-1X8 (Cl^-), 400 mesh, by a

linear NaCl gradient elution. The elution profiles are shown in Fig.19. In each case nearly identical elution patterns of 6 phosphorus inositol containing peaks were obtained. These peaks were eluted at 0.18 M, 0.36M,0.42M, 0.54M, 0.64M and 0.72M-NaCl concentrations and were designated as I,II,III,IV,V and VI corresponding to elution order. The molar ratios of phosphorus: myoinositol (P/MI) are summarised in Table 12. These results confirm the presence of mono-,di-, tri-,tetra-,penta- and hexaphosphates of myoinositol. In addition the data also shows the occurrance of stepwise dephosphorylation of phytic acid *in vitro*.

Although elution profiles of myoinositol phosphate intermediates produced by different phytases appear identical, as far as the number of peaks are concerned, the mole fraction of component intermediates are characteristically different indicating the distinct nature of different phytase species. For instance, the results shown in Fig.21 show that all forms of phytase do not favour the hydrolysis of inositol monophosphate except phytase II. In other words phytase II appear to dephosphorylate the monophosphate ester of myoinositol at a faster rate. Another striking difference is that phytase II hydrolyses myoinositol diphosphate less rapidly than the remaining forms of phytase. Enzyme IA dephosphorylates pentaphosphate less rapidly than all other forms of phytase. Enzyme IE, similarly dephosphorylates the triphosphate less rapidly than others. In other words there is a significant difference in the rate of hydrolysis of intermediates when present together. It may be pointed out

however, that these results have to be viewed differently than those reported in previous chapter because in the case of latter the purified single substrate was used instead of mixture. The overall rate of hydrolysis of phytic acid by different phytase forms is given in Table-11. These results show that rate of hydrolysis with enzyme IA and II are relatively lower than the remaining forms of enzyme. Taking all these results together it seems clear that although the dephosphorylation of phytic acid is stepwise, the production of various intermediates, occurs differently. These observations are in agreement with those reported by Biswas et al(10). But these observations do not exactly tell us the point of difference in the pathway. For this purpose the individual intermediates yielded during hydrolysis by various phytase enzymes have to be identified.

5.3.2 Identification of Myoinositol Phosphate Intermediates

The identification of the intermediates isolated above were further fractionated and identified by paper chromatography , IR spectroscopy and by chemical degradative method using standard myoinositol phosphates of known structure

The paper chromatograph showing the separation of various phosphate intermediates is shown in Fig.20 and the R_f values with respect to phytic acid are given in Table 13. These results demonstrate that various inositol phosphates were easily resolvable. However, on the basis of R_f values, isomeric forms of myoinositol phosphates could not be identified, as the difference in the R_f value was too small to

reflect any structural difference. Different inositol phosphates were isolated and subjected to periodate oxidation and reduction with $NaBH_4$ to yield phosphorylated sugar alcohols. These sugar alcohols were dephosphorylated in acid medium and identified by IR spectroscopy using known standards of sugar alcohols. The IR spectra of standard sugar alcohols have been shown separately under Appendix

In order to confirm the results, cis-OH acid migration in myoinositol polyphosphates was also carried out followed by periodate oxidation and reduction. On the basis of sugar alcohols produced the possible structures of myoinositol phosphates were worked out as shown in Fig.22. A consolidated summary of the results of various experiments leading to the identification of myoinositol phosphate intermediates formed during the hydrolysis of phytic acid by various phytases is given in Table.14. Sugar alcohols obtained from the myoinositol tetraphosphate, formed as an intermediate in dephospyorylation of phytic acid by different species of phytase was identified as altritol in all the phytase hydrolyzates. No acid catalysed cis-OH group migration occured. Thus as shown in Fig.22 myoinositol 1,2,3,4 tetraphosphate is the only possible isomer formed in all cases. Sugar derivative of myoinositol triphosphate, formed by phytase I isoenzymes, was found to be ribitol. Also there was no cis migration. This indicates that the myoinositol 1,2,3 triphosphate is formed in hydrolysis of phytic acid by phytase I isoenzymes (Fig.22b). But the sugar derivative of inositol triphosphate formed by phytase II was found to

be D-arabitol (Fig.22c). By cis-OH migration of phosphate group, it yielded sorbitol. Thus, the structure of inositol triphosphate formed in phytase II hydrolysis, is myoinositol-2,3,4-triphosphate (Fig.22c). Sugar ddrivative of inositol diphosphate was found to be erythritol in all cases. Cis migration of phosphate group, resulted in formation of D-arabitol (Fig.22d), thus, the myoinositol diphosphate formed by all the phytases is inositol-2,3-diphosphate.

Identification of myoinositol pentaphosphate(s) produced during phytic acid hydrolysis by enzyme has been somewhat uncertain. The ^{31}P NMR data (Fig.23) and the paper chromatographic analysis show that only one type of penta-phosphate ester of myoinositol is formed as similar observa-tions were obtained in the case of all isoenzymes. On the basis of the myoinositol 1,2,3,4 tetraphosphate intermediate , there could be either myoinositol 1,2,3,4,6 pentaphosphate or myoinositol 1,2,3,4,5 pentaphosphate. If we go by the general pattern of plant phytase action as suggested by Cosgrove(92) that phytase action starts by catalysing the hydrolysis of phosphate group on position 6 of phytic acid molecule, then the myoinositol pentaphosphate intermediate should be predominantly myoinositol-1,2,3,4,5 pentaphosphate.

The electrophoretic mobility, stability to cis migration condition and the isomerization pattern produced under trans migration conditions and optical activity are exactly same as reported by Lim and Tate(75) for the myoinositol 1,2,3,4,5 pentaphosphate produced by crude wheat bran phytase degrada-tion of phytic acid. Thus, in all probabilityit seems that

myoinositol 1,2,3,4,5 penta-phosphate is the pentaphosphate intermediate formed as the first step of hydrolysis of phytate molecule by phytase species *in vitro*.

The dephosphorylation pathways given in Fig.24 clearly show that the mode of action of phytase I isoenzymes *in vitro* is identical. In other words in isolated systems using purified enzymes and pure inositol hexaphosphate as substrate the stepwise dephosphorylation proceeds via identical lower phosphates. As pointed out earlier, the only difference appears to be in the rate of dephosphorylation. In contrast the low molecular weight phytase II follows a different pathway in the respect that upto tetraphosphate, the pathway is identical with those of phytase I isoenzymes but at tetraphosphate level it is different catalysing the hydrolysis of phosphate group on position no.1 instead of position no.4. Hence myoinositol 2, 3,4 instead of myoinostiol 1,2,3 triphosphate intermediate is formed. Beyond this point again the pathway of phytase II is identical with those of phytase I isoenzymes. Although the present study has established the mode of action of various phytase forms *in vitro*, it cannot be said with certainty that the same is true for the *in vivo* process especially because in the latter case all the forms of phytase and also all the lower phosphates may be present at the same time . Hence the situation is much more complex and the results *in vitro* only give limited information regarding the actual role of various forms of phytases present. In the situation mentioned above it is not difficult to vis-

ualise that the controlled dephosphorylation of phytic acid by various phytases when present together will be decided by the relative concentration of various phytase species, by the relative affinity of various enzymes towards phytic acid and lower inositol phosphate intermediates. It therefore appears that in order to understand the role of different phytase species in the phytic acid metabolism, it would be essential to know the level of activities of various phytases and also the relative concentrations of various inositol phosphates at each stage. In order to resolve the above question the mode of development of different phytase species has been studied and results correlated with the substrate affinities and other kinetic parameters. The results of this study are presented in the next chapter.

5.4 SUMMARY

The myoinositol polyphosphates produced during the *in intro* hydrolysis of phytate molecule by various forms of phytase were identified and the dephosphorylation pathways for each form of the enzyme were established. It was found that high molecular forms of phytase (phytase IA, IB,IC,ID and IE) follow the same dephosphorylation scheme whereas the low molecular form functions differently. The main difference is in the dephosphorylation of myoinositol 1,2,3,4-tetra phosphate intermediate. The high molecular phytase species catalyse the hydrolysis of phosphate monoester bond at position 4 while the low molecular form (phytase II) catalyses the hydrolysis of phosphate monoester bond at position 1 in

phytic acid molecule. In addition, there is a significant difference in the rate of hydrolysis of phytic acid and the relative concentration (mole percent) of various myoinositol polyphosphate intermediates yielded by different forms of phytase.

TABLE-11: PERCENT HYDROLYSIS OF PHYTIC ACID BY VARIOUS FORMS OF PHYTASE

Phytase form	Phytate Hydrolysis[1] (%)
IA	76.7
IB	85.7
IC	85.6
ID	90.4
IE	87.8
II	53.8

1 Percent hydrolysis was calculated by estimating the total myoinositol content in the combined myoinositol phosphate intermediates produced during 48 hr of hydrolysis under standard assay condition.

TABLE-12: PHOSPHATE/INOSITOL RATIO DETERMINATION IN PEAKS OF DOWEX 1x8 (Cl^-) CHROMATOGRAPHY OF HYDROLYZATES OF DIFFERENT PHYTASE ISOENZYMES

	Phytase Isoenzymes																	
	IA			IB			IC			ID			IE			II		
Peak No.	P_i	MI	Ratio P_i/MI	P_i	MI	Ratio P_i/MI	P_i	MI	Ratio P_i/MI	P_i	MI	Ratio P_i/MI	P_i	MI	Ratio P_i/MI	P_i	MI	Ratio P_i/MI
I	24	25	1.0	30	29	1.0	29	30	1.0	35	37	1.0	36	36.0	1.0	14	15.0	0.9
II	16	7.5	2.1	29	14.6	2.0	25	12.4	2.0	24	13	1.9	10	5.0	2.0	27	13.6	2.0
III	25	8.0	3.1	19	6.2	3.1	14	4.7	3.0	12	3.8	3.2	23	7.7	3.0	11	3.8	2.9
IV	20	5.0	4.0	15	3.8	4.0	10	2.4	4.2	10	2.4	4.2	25	6.2	4.0	11	2.7	4.1
V	30	6.2	4.8	24	4.7	5.1	29	5.8	5.0	25	5.0	5.0	25	5.0	5.0	24	4.7	5.1
VI	26	4.2	6.1	37	6.2	6.0	27	4.4	6.1	34	5.7	6.0	16	2.7	5.9	29	4.7	6.2

P_i - Inorganic phosphorus

MI - Myoinositol

TABLE-13: Rf VALUES OF MYOINOSITOL POLYPHOSPHATES IN PAPER CHROMATOGRAPHY

Phytase Iso-enzyme	*Rf values of Myoinositol Phosphates					
	MIP_1	MIP_2	MIP_3	MIP_4	MIP_5	MIP_6
IA	2.00	1.80	1.60	1.41	1.20	1.00
IP	2.04	1.80	1.62	1.40	1.20	1.00
IC	2.00	1.80	1.60	1.40	1.23	1.02
ID	2.03	1.82	1.63	1.40	1.20	1.01
IE	2.03	1.81	1.60	1.42	1.22	1.04
1I	2.00	1.80	1.61	1.44	1.20	1.00

*Rf = $\frac{\text{Distance migrated by inositol polyphosphate}}{\text{Distance migrated by phytic acid}}$

MIP = Myoinositol phosphate ester

TABLE-14: IDENTIFICATION OF MYOINOSITOL POLYPHOSPHATES BY PERIODATE OXIDATION REDUCTION METHOD

Phytase Enzyme	Myoinositol Tetraphosphate (IP_4) Sugar Derivative		Derived Structure of IP_4	Myoinositol Triphosphate (IP_3) Sugar Derivative		Derived Structure of IP_3	Myoinositol Diphosphate (IP_2) Sugar Derivative		Derived Structure of IP_2
		Cis CH acid Migration			Cis OH acid Migration			Cis OH acid Migration	
IA	Altritol	-	1,2,3,4	Ribitol	-	1,2,3	Erythritol	Arabitol	2,3
IB	-do-	-	1,2,3,4	-do-	-	1,2,3	-do-	-do-	2,3
IC	-do-	-	1,2,3,4	-do-	-	1,2,3	-do-	-do-	2,3
ID	-do-	-	1,2,3,4	-do-	-	1,2,3	-do-	-do-	2,3
IE	-do-	-	1,2,3,4	-do-	-	1,2,3	-do-	-do-	2,3
II	-do-	-	1,2,3,4	-do-	Sorbitol	2,3,4	-do-	-do-	2,3

TABLE-15: PROPERTIES OF MYOINOSITOL PENTA PHOSPHATE

Property	Myoinositol Pentaphosphate		
	Sample	Standard	
		P-1,2,3,4,5	P-1,2,3,4,6
*Electrophoretic mobility.	3.75	3.70	3.60
**$(M)_D^{25}$	+9±5	+9±4	0± 13

*Electrophoretic mobility was measured by high voltage paper electrophoresis.

**$(M)_D^{25}$: Molar optical rotation, measured by visual polarimeter using sodium D-line as light source.

Fig. 19 : Isolation of Inositol Phosphate Intermediates from Hydrolyzates of Phytase Isoenzymes by Dowex-1x8(Cl^-) Column Chromatography.

Mixture of inositol phosphate intermediates formed after hydrolysis of sodium phytate by each phytase isoenzyme was separated by Dowex-1x8(Cl^-) column chromatography. Elution of various inositol phosphates was carried out by 0-1M NaCl gradient at a rate of 0.5 ml/min. 100 fractions, each of 5 ml were collected. 1 ml of each fraction was tested for inositol phosphate by Bartlett method (176) as described in the text. The elution profiles of inositol phosphates from hydrolyzates of phytase IA, IB, IC, ID, IE, and II are represented in this figure.

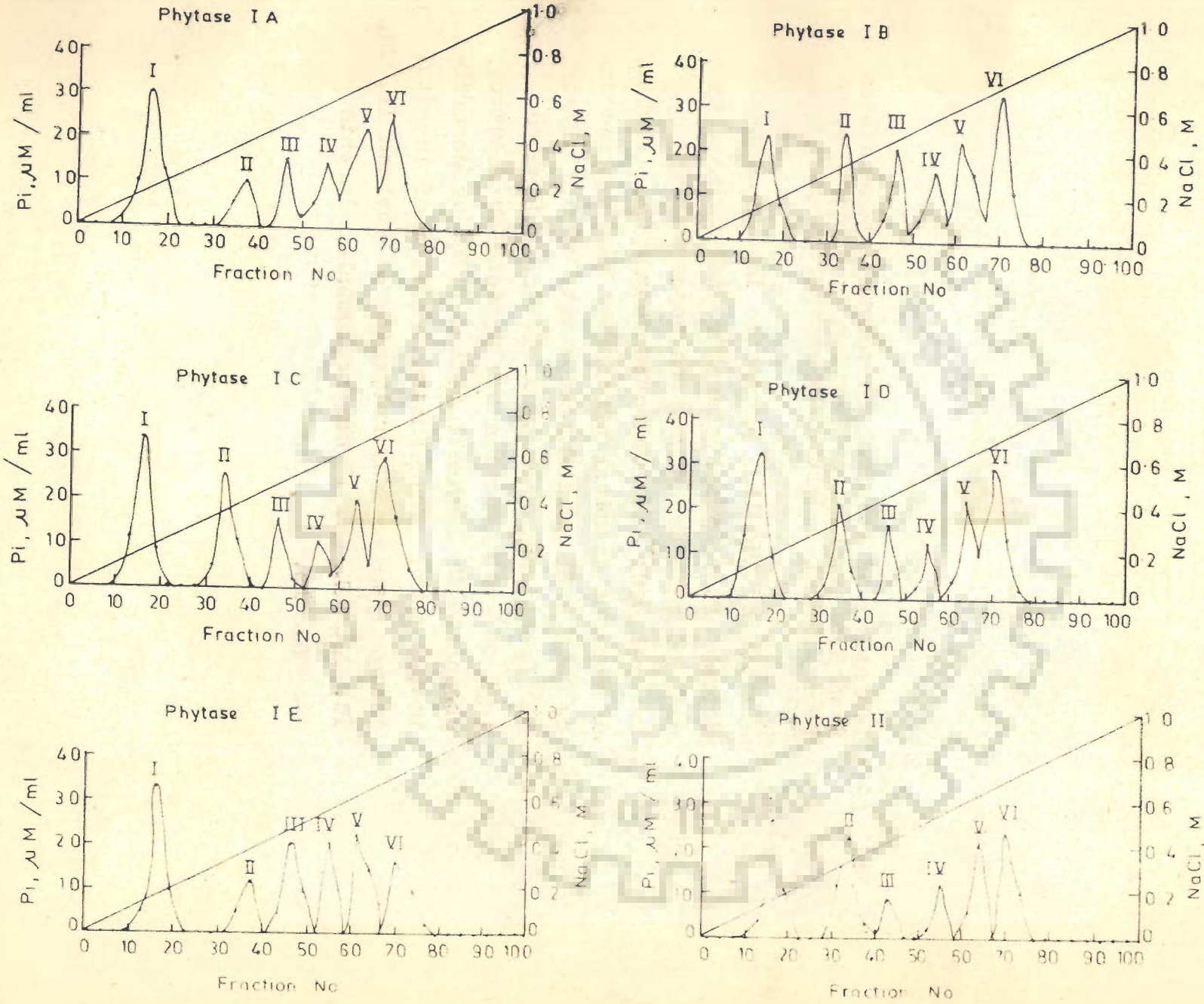


Fig. 19 - Dowex 1X8 (Cl^-) Column Chromatography of Inositol Phosphates Formed by Hydrolysis of Phytate by Different

Fig. 20 : Ascending Paper Chromatography of Various Myoinositol Phosphate Esters.

Paper chromatography of different myoinositol phosphate esters isolated from Dowex AG-1x8(Cl^-) column, was carried out on Whatman No.1 filter paper using 2-propanol-NH_3-H_2O (5:4:1) as the solvent system. The paper chromatogram containing myoinositol phosphate esters and a standard of myoinositol hexaphosphate, was developed in this solvent for 36 hrs at 30°C. The myoinositol phosphate esters were located by spraying the chromatogram with Axelrod and Bandurski reagent followed by U.V. exposure. Spots marked as I, II, III, IV, V, and VI represent the myoinositol mono-, di-, tri-, tetra-, penta- end hexa phosphates. MHP represents the standard myoinositol hexaphosphate.

Fig. 21 : Relative Mole Percentages of Myoinositol Phosphate Intermediates formed During Hydrolysis of Phytic Acid by Various Phytase Isoenzymes.

Various myoinositol phosphates mono-(MIP_1), di-(MIP_2), tri-(MIP_3), tetra-(MIP_4) and penta-(MIP_5) phosphates formed during hydrolysis of phytic acid (MIP_6) by various phytase isoenzymes were obtained from Dowex-1x8(Cl^-) column chromatography of their hydrolyzates. Their relative concentrations are shown in this figure.

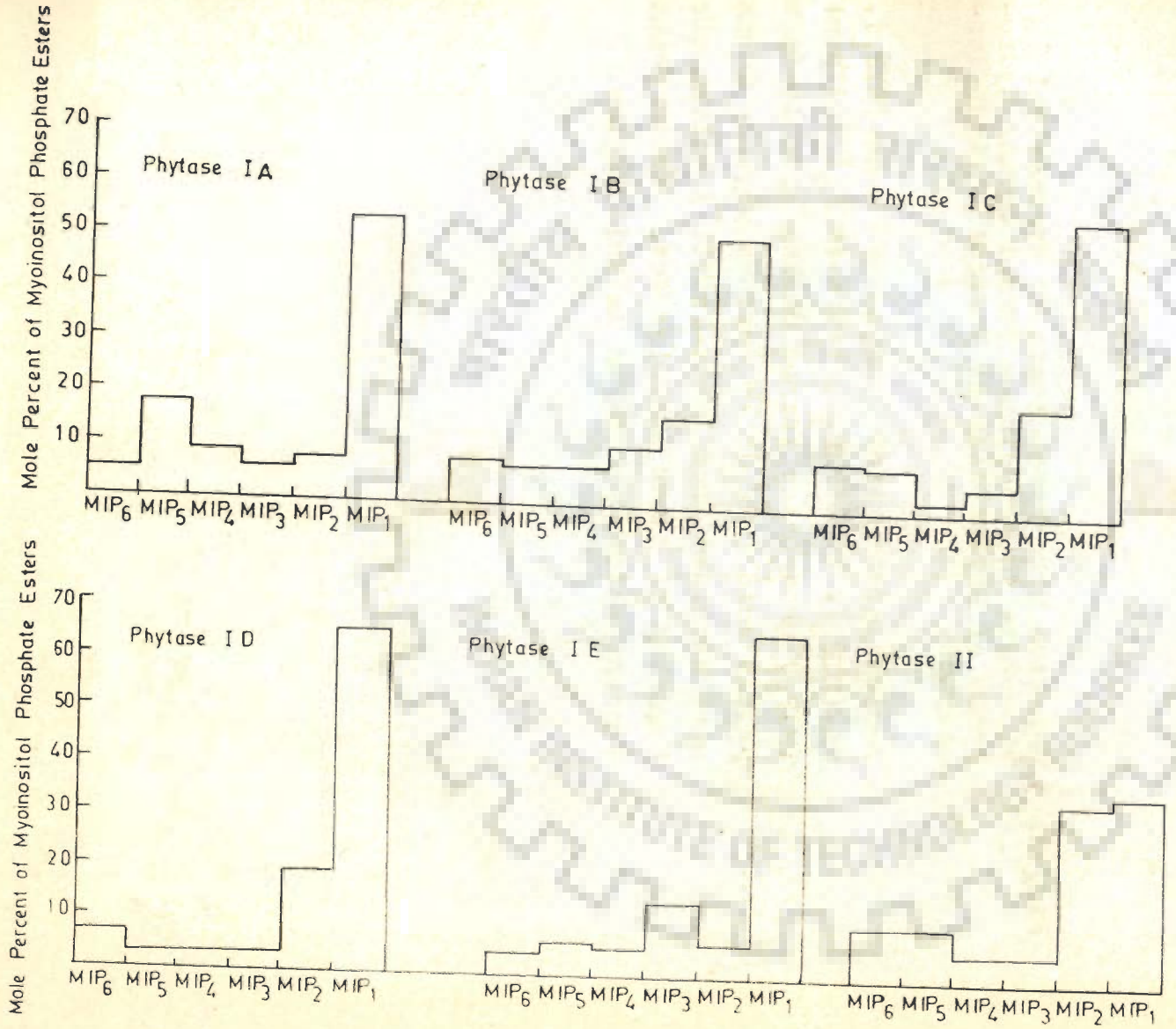


Fig 21 - Relative Mole Percentages of Myoinositol Phosphate Intermediates in Reaction Hydrolyzates of Phytases

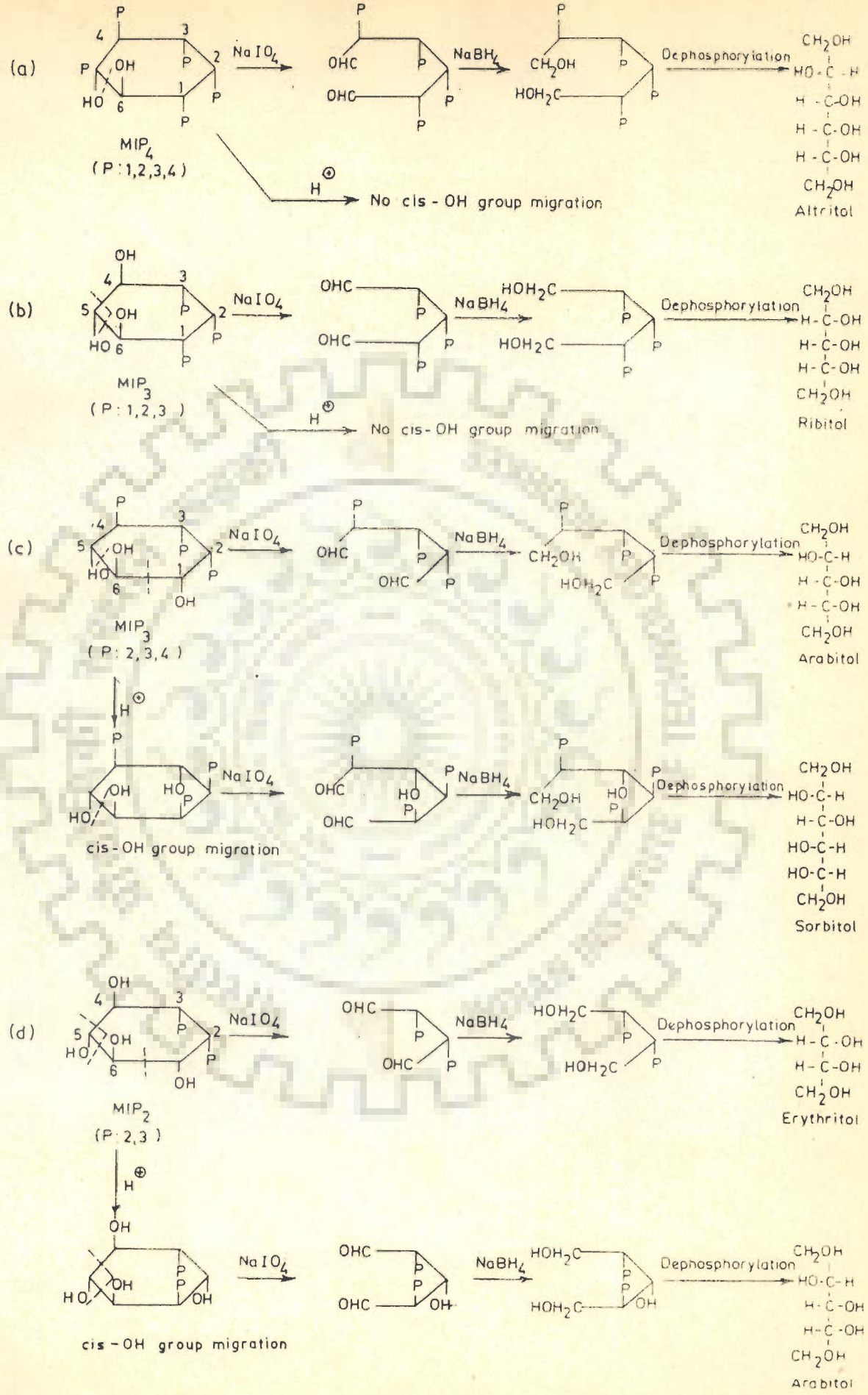


Fig 22 - Sugar Alcohols Formed by Periodate Oxidation of Myoinositol

Fig. 23 : ^{31}P NMR Spectrum of Myoinositol Pentaphosphate

Various myoinositol phosphates produced during the hydrolysis of phytic acid by individual phytase isoenzymes were isolated and identified by different techniques given in text. Sodium salts of myoinositol pentaphosphate intermediates were dissolved in distilled water. Their ^{31}P NMR spectroscopy was carried out at 40.25 MHz. ^{31}P NMR spectrum of myoinositol pentaphosphate produced during hydrolysis of phytic acid by phytase IA is shown here. In the case of enzymes IB, IC, ID, IE and II, identical ^{31}P NMR spectra were observed.

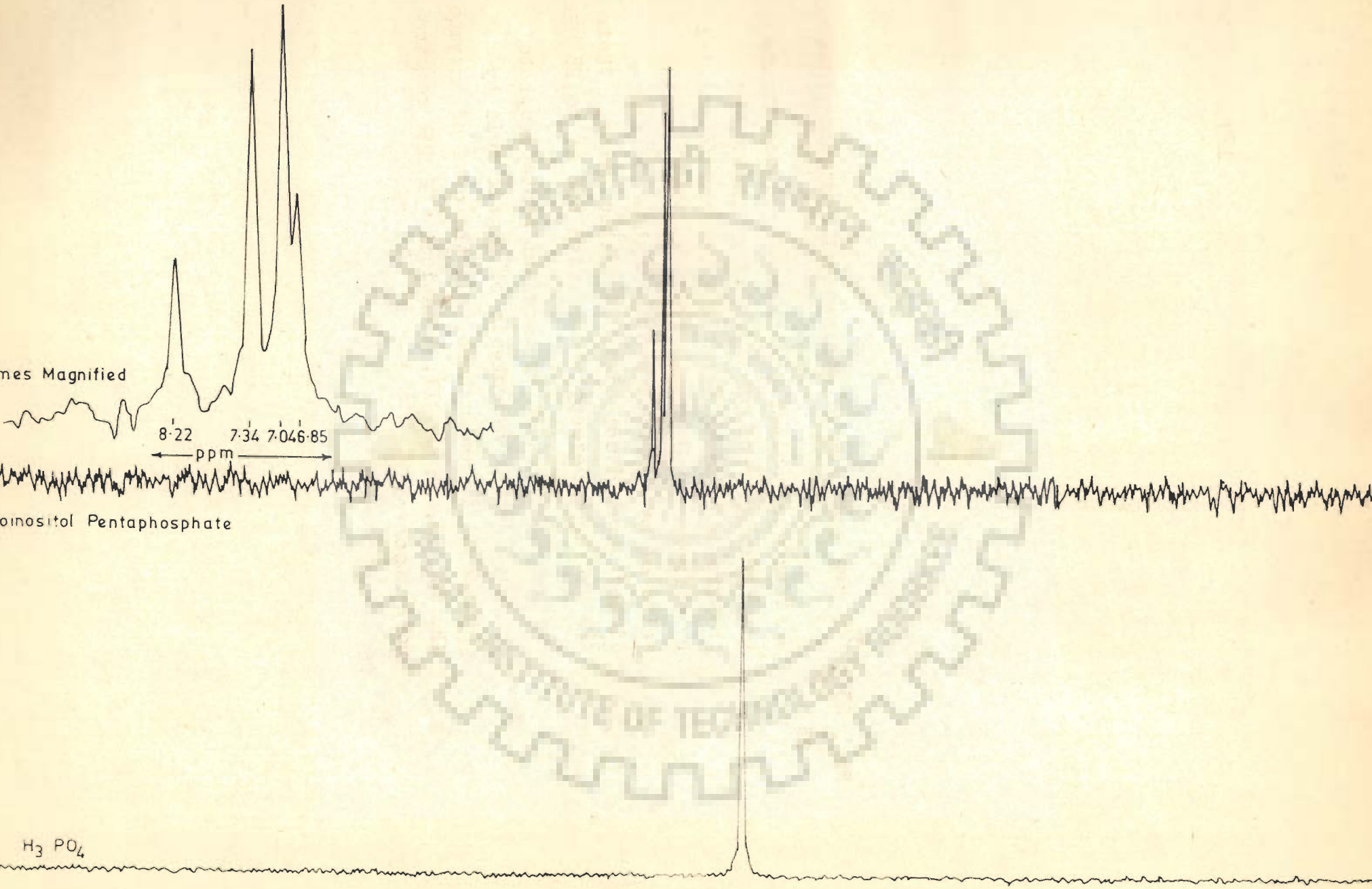


Fig 23 - ^{31}P NMR SPECTRUM

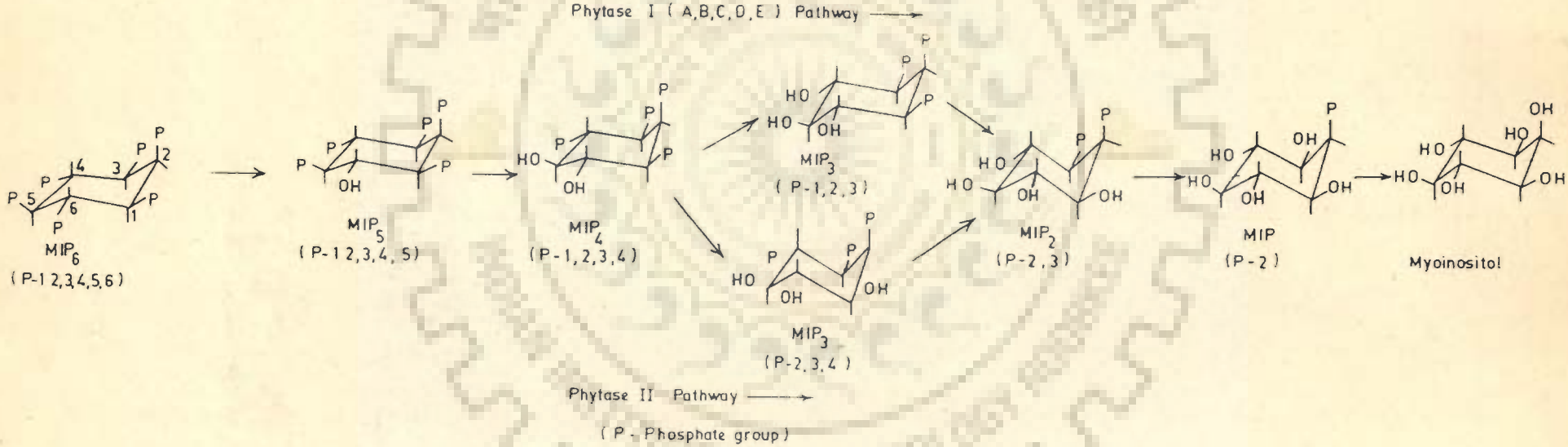


Fig 24 - Dephosphorylation Pathway of Phytic Acid

CHAPTER - VI

DEVELOPMENT OF MULTIMOLECULAR FORMS OF PHYTASE IN GERMINATING COTYLEDONS OF *CUCURBITA MAXIMA*

6.1 INTRODUCTION

Phytase which catalyses the hydrolysis of myoinositol hexaphosphate (phytic acid) and thus falicitating the mobilisation of the primary reserve of organic phosphate in the germinating seedlings (148-150), has been found missing from the cotyledons of dormant seeds. But its *de novo* synthesis starts 12-36 hours after the imbibition of seeds (86,100). In previous chapters we have reported the results showing the involvement of multiple forms of phytase in the degradation of phytic acid in *Cucurbita maxima* seedlings. These observations were in agreement with those reported for wheat and rice (74-76). The properties of different isoenzymes of phytase especially the substrate specificity with varying degree of affinity towards phytate and lower polyphosphates of myoinositol reported in Chapter IV and V have suggested that the regulation of the dephosphorylation of phytic acid and hence the mobilisation of stored phosphorus from cotyledons to other parts of the growing plant during early period of germination is determined by the relative levels of both the different isoenzymes of phytase and the lower polyphosphates of myoinositol. Unfortunately, no information on the development of the multiple forms of phytase is available. Consequently the physiological significance and role

of the multi molecular forms of phytase in growing embryo still remain unassigned. In the present chapter we have reported the results of the development of six multimolecular species of phytase and their physiological role in Cucurbita maxima seedlings.

6.2 MATERIALS

Cycloheximide was obtained from the Sigma Chemical Co., St. Louis, (USA). All other chemicals and materials used were same as reported in Chapter III unless mentioned otherwise.

6.3 METHODS

In order to study the developmental pattern of phytases in Cucurbita maxima cotyledons during germination, the Cucurbita maxima seeds were germinated for a period of 372 hours. Germination was done as described in Chapter III. One hundred cotyledons were picked up at randomly from each stage of development of embryo indicated and phytase enzyme was extracted as before. The various forms of phytase enzyme were separated and resolved by Sephadex G-150 gel filtration and CM cellulose column chromatography. All the procedures of extraction, isolation, purification and enzyme assay were carried out in the same manner as given in Chapter III, unless stated otherwise.

6.3.1 Seed Germination in Presence of Cycloheximide

For studying the development of phytases the cycloheximide treatment of seeds was done. Cucurbita maxima seeds

were first soaked in distilled water for 12 hours to allow the initiation of germination and then washed with 50×10^{-6} M cycloheximide solution in distilled water. The seeds were allowed to germinate in dark for indicated periods. One hundred seedlings were picked up at randomly at each stage of germination and the enzymes were analysed by Sephadex G-150 filtration and CM cellulose column chromatography as described previously.

6.4 RESULTS

6.4.1 Development of Phytase System in Germinating Cotyledons of Cucurbita Maxima

Seeds of Cucurbita maxima were imbibed in water and total phytase activity was assayed. Results shown in Fig.25, show that the dormant seeds are devoid of phytase activity, but its de novo synthesis occurs within 12 hours of imbibition. Once the initial lag period of about 6 hour is over the rate of biosynthesis of phytase increases rapidly reaching a maximum level after about 6 days time at 35°C. The peak period stays upto 9th day before starting to decline. Obviously, the period between 6 to 9 days after imbibition of seeds represents the period of highest metabolic activity in pumpkin seeds requiring the reserve phosphate (phytate) to be mobilised at a much faster rate in order to meet the phosphorus requirements for the growing plant.

In order to investigate the developmental pattern of the different molecular forms of phytase, the high and low

molecular forms of phytase 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 phytase I and II in germinating cotyledons of Cucurbita maxima (Fig. 26). It was found that during the first 12 hours after imbibition, only the high molecular phytase (phytase I) was synthesized and there was no synthesis of the low molecular weight enzyme, phytase II, during this period. In fact, phytase II started appearing at about 36 hours of germination. Its activity level was very low in the beginning but which unlike phytase I gradually went on increasing throughout the entire 15 days period of germination. The level of phytase I on the other hand increased upto 7th day and declined quite rapidly. It is interesting to note that the low molecular phytase species which were less dominant in initial stages (upto 8th day) became highly dominant in the later half of germination. Relative proportions of phytase I and II in cotyledons at different stages of germination are summerised in Table 16. Although there is a concomitant fall in the proportion of phytase I level with the elevation of phytase II, at the moment it is uncertain if there is at all any correlation between the disappearance of high molecular form and the appearance of the low molecular form phytase in germinating cotyledons. Nevertheless the physiological roles of phytase I and II appear to be different and important.

Since phytase I fraction was further separated into

5 species by CM cellulose, the development of each species was investigated. The phytase I species isolated from germinating cotyledons of different stages were resolved on a CM cellulose column as described previously and the relative levels of activity were taken. Measure of de novo synthesis (Fig. 27) shows the elution profiles of phytase I isoenzymes from the CM cellulose column. The data of the relative activity of each phytase I forms during the germination period are given in Table 17.

It was observed that during first 12 hours of germination, the isoenzyme IB was found to account for more than half of total phytase I activity followed by phytase IA. However, the activity level of IB enzyme declined rapidly and became insignificant in about 48 hours of germination. The activity level of IA increased upto 48 hours contributing about 50 % of total phytase I activity. This state was maintained upto 180 hours. After this period, the level of its activity started declining slowly. The enzyme IE was found to follow yet another developmental pattern as its activity increased progressively from 10 % (after 12 hours) to 70 % of phytase I at the end of 372 hours of germination. Enzyme IC and ID showed minor activities. The activity of IC and ID were maximum at 84 and 48 hours of germination respectively. After that they started declining and became insignificant by the end of germination period (15 days).

6.4.2 Effect of Cycloheximide on the Biosynthesis of Different Phytase Isoenzymes

The effect of cycloheximide, a strong inhibitor of

protein biosynthesis, on the biosynthesis of isoenzymes of phytase has been investigated by determining the levels of various isoenzymes during a specified period of germination. For this purpose nearly 100 germinating cotyledons were at randomly picked up and phytase activity was determined. The data summarised in Table 18 shows the effect of cycloheximide on the synthesis of total phytase at different stages of germination. Since cycloheximide was applied only after 12 hours of imbibition period, the level of phytase enzyme at this stage was taken as 100 %. During this period 92 units/ 100 cotyledons were synthesized in the untreated seeds. The treated seeds showed no activity at all, however, during the period 12-36 hours there was a net synthesis of 102 units of phytase in the control where as in the treated ones only 64 units were synthesized. Thereby an inhibition of 37 % occurred. During the period 36-84 hours only 123 units were synthesized and surprisingly no activity was found in the treated ones i.e. a 100 % inhibition of phytase activity was found. Similarly between the period 84-180 hours there was little synthesis of phytase enzyme in the control and complete absence of activity in the treated ones. These results therefore indicate that cycloheximide inhibits the de novo synthesis of phytase in Cucurbita cotyledons.

In order to find out the effect of cycloheximide on the synthesis of various phytase species, the phytase species were separated both on molecular weight and on the basis of ionic properties. For the former Sephadex gel filtration was

used. Fig. 29 shows the elution profiles of high and low molecular weight phytases at different stages of germination in the presence of cycloheximide. Fig. 30 shows the elution profiles of isoenzymes of phytase I from CM cellulose column. Data summarised in Table 19 shows that phytase II (low molecular weight form) show a synthesis of 6 units/100 cotyledons between 12-36 hours of germination. During the same period the treated ones show only 2 units. Thus there is 66.7 %. inhibition of synthesis of low molecular weight phytase. During this period total units of phytase IA, IB, IC, ID and IE synthesized are 39, 43, 6, 7, 26 units respectively. In the treated cotyledons on the otherhand the activity levels of these enzymes are 13,15,4,2,12 units respectively. Thus in each case the inhibition of enzyme synthesis is significant. However, the inhibition of phytase IA, IB and ID was maximum. Phytase IE showed only 46 %. inhibition. Between germination period of 36-84 hours the inhibition of phytase IA, IB and IE was total. In fact during this period even in the control the level of phytase ID was negligible. The inhibition of synthesis of phytase II was about 95 %.. In the third period of germination between 84 and 180 hours only enzyme II was synthesized. The cycloheximide treatment stopped the synthesis of all the phytase I enzymes. In the case of low molecular weight enzyme (phytase II) nearly three-fourth of synthesis was inhibited by cycloheximide.

Fig. 31 shows a comparision of the synthesis of various phytase isoenzymes at various stages of germination both in

the presence and the absence of cycloheximide. Since the inhibition of phytase II synthesis was not complete even during later stages of germination it is assumed that some high molecular form of the enzyme was converted to low molecular form. This is reflected from the fact that the inhibition of synthesis of phytase II during 84-180 hours was 77 % and the decrease in inhibition would mean that some high molecular weight form of phytase has been converted into the low molecular weight form.

6.5 DISCUSSION

The present study was undertaken to investigate the following aspects of phytic acid metabolism in germinating seeds of Cucurbita maxima.

(1) The mode of development of various forms of phytase

(2) Their physiological role and relative contributions in the mobilisation of phytate phosphorus during germination.

The mode of formation of phytases in phytase system was determined by measuring the changes in the levels of activities of various forms of phytase as well as total phytase over 15 days germination period. In general, the pattern of total phytase activity in pumpkin seeds was in agreement with that observed in other seeds i.e. phytase was not present in the dormant seeds and its de novo synthesis occurs only after 6 hours imbibition of seeds in water (100). Thus increase in

phytase activity in cotyledons following the initial lag of about 12 hours indicates the rapid mobilisation of reserved phosphorus (phytate phosphorus) during germination.

For the sake of convenience, we can divide the whole period of germination into three phases. Phase I represents the initial phase in which the concentration of phytic acid is high and the enzyme is either present in low concentration or absent completely. This phase seems to cover first 36 hours of the germination period. In this phase mostly the endogeneous inorganic phosphorus and higher polyphosphates of inositol may be expected to be present besides high concentration of phytate. The II phase, which represents the phase of maximum metabolic activity ccvering a period of 36 hours to nearly 180 hours is expected to require maximum amount of inorganic phosphorus to meet its energy requirement. During this period the phytic acid molecule must be efficiently degraded to release or to satisfy the requirement of phosphorus of the growing plant. In the III phase, the stock of reserved material in the cotyledons will be reduced to low level and much less amount of unhydrolysed phytic acid will be present. In addition, in this stage lower insitol phosphates produced as a result of fast enzymatic activity may be present. In other words, the phosphate requirement of the growing embryo in the absence of exogeneous supply of phsphorus as in the present study, will be satisfied by further hydrolysis of lower inositol phosphates by the phytase enzymes.

In view of these 3 phases and on the basis of

developmental pattern, attempt has been made to assign the physiological role of various molecular forms of phytase.

Fig. 28 summarises the developmental pattern of various forms of phytase as a function of time. The information given in this figure clearly shows that none of the phytase forms was present in dormant seeds. But after 12 hours imbibition a series of phytase species were synthesized in the germinating cotyledons of Cucurbita maxima. It is worth to note that the low molecular weight enzyme II is completely absent at this stage. Similarly the total contribution of the enzyme IC, ID and IE is less than 20 %. Thus apparently enzyme II and phytase IC, ID and IE do not play important role in this I phase of germination. In other words they may not act on the phytate molecule sufficiently whereas enzyme IA and IB contribute over 60 % of total phytase activity at this stage indicating that these two enzymes are important in the mobilisation of phytate phosphorus in the early phase of germination. Out of these two enzymes, IB shows very interesting behaviour. At 12 hours of germination period, its concentration is highest which then declines from 40 % to nearly less than 5 % in next 36 hours. These results are interpreted to mean that the sudden increase in the level of enzyme IB is of physiological significance in releasing the inorganic phosphate from phytic acid. If this observation were true then one would expect that sufficient concentration of penta-phosphate ester of inositol would accumulate which may supress the further synthesis of enzyme IB.

The developmental pattern after 36 hours throw another interesting light on the possible physiological role of phytase enzymes. Here low molecular weight enzyme has started appearing though its level is below 5 %. The enzyme IE level increases from 8 % to nearly 20 %. There is not much difference in level of enzyme IA and IB. As pointed out earlier, the enzyme IB level decreased significantly with concurrent increase in IA level. Thus at this stage if we go only by relative levels of different species of phytases, we can surely say that at 36 hour period which is a border line physiological phase between I phase and II phase, only enzyme IA, IB and IE play important role. Again, if we go from the previous consideration i.e. at this stage inositol penta-phosphate and tetraphosphates are produced, then enzyme IE and IA may contribute significantly to the hydrolysis of myoinositol pentaphosphate. At the moment we do not have a definite proof to support the hypothesis but the relative substrate specificity and affinity of various phytases towards different inositol phosphate intermediates support the above point of view.

At 48 hours which is the beginning of the II phase, we see a very difficult pattern than what was observed at 36 hours. For instance, the level of enzyme IA activity was highest at this time, contributing nearly 35 % of the total activity and enzyme ID, IE and II also become significant, contributing 12, 18 and 20% respectively, unlike at 12 hours and 36 hours of germination when the level of enzyme IB was

almost insignificant. The level of enzyme IC is also insignificant. The level of IC enzyme has decreased progressively between 12-48 hours of germination. If at this stage, we assume that lot of lower inositol phosphates were present compared to hexaphosphate and pentaphosphate, it can be said that enzymes ID, IE and II were responsible for hydrolysis of the lower inositol phosphates. The enzyme IA can be considered to be active towards higher inositol phosphates including hexaphosphate since its level increases upto 48 hours and then starts declining.

At 84 hours another interesting pattern of development of phytase system was observed. At this hour the phytase IA and IE enzyme activity were nearly same as at 48 hours but the level of activity of ID decreased to nearly two thirds of the 48 hours activity while the activity of IC and II increased quite significantly. On the basis of these results we can say that the major contribution i.e. about 60 % comes from enzymes IC, IE and II correlating the previous observation.

It can therefore, be concluded that IC, IE and II enzymes significantly contribute to the hydrolysis of lower inositol phoaphate intermediates(MIP_3, MIP_2, MIP_1) which are produced during the hydrolysis of phytic acid while the phytase ID and IB do not play any significant role. In other words the developmental pattern of phytase isoenzymes during germination period of 84-180 hours, remains nearly unchanged with slight increase in enzyme II level. These observations indicate, therefore, that the relative concentrations of substrates

between 84 hours and 180 hours of germination undergo little change. This phase as pointed out above is the phase of maximum metabolic activity.

In the III phase at 228 hours, there is once again a dramatic change in the pattern of synthesis of various phytases. At 228 hours nearly 2 fold increase takes place in the level of phytase II enzyme as compared to the 84 hour level. The activity level of all enzymes declines substantially. In fact the enzymes IB, IC, ID become insignificant and enzyme IA which played a major role in early phases becomes a minor enzyme. The level of enzyme IE stays nearly constant throughout the phase III.

The level of enzyme II rises further during 300 to 372 hours of germination. At the last stage i.e. at 372 hours, the level of enzymes IA, IB, IC and ID becomes insignificant while the most dominating enzyme is phytase II, contributing nearly 70 % of total phytase activity. The enzyme IE contributes only about 20 % . Considering this pattern of synthesis of various enzymes we can say that enzyme IA and IB are important in initial stages and enzyme II and IE are important in later stages.

It may be pointed out, however, that <u>in vitro</u> the conditions of phytate degradation by enzyme may be entirely different than those prevailent <u>in vivo</u>. In the former case the enzyme, substrate and the products remain in the closed system whereas they are not so <u>in vivo</u>. Nevertheless the data indicates that <u>in vitro</u> in isolated system, all forms of

phytase are capable of hydrolysing phytic acid completely though with varying efficiency. But when the enzyme, substrate and the products - all are present together *in vivo*, the affinity of the enzyme towards the substrate and the relative concentration of the enzyme and the substrate would determine the action of different phytase species. In other words which of the species would hydrolyse which phosphate ester bond in phytic acid and which of the lower phosphates, will depend upon and controlled by the above factors.

On the basis of kinetic data and the pattern of development of the different phytase isoenzymes, the possible role of each phytase isoenzyme in the course of phytate dephosphorylation *in vivo* has been suggested (Fig. 32). For instance, phytase IB is involved in the selective dephosphorylation of MIP_6 at position No. 6 to yield corresponding MIP_5. Enzyme IE appears preferentially to catalyse the dephosphorylation of pentaphosphate at position No. 5 to yield tetraphosphate 1,2,3,4 - MIP_4. Enzyme ID and II appear to favour the hydrolysis of phosphate monoester bond at position 4 and 1 in MIP_4, respectively to give two IP_3 (1,2,3-IP_3 and 2,3,4-IP_3). Triphosphate ester 1,2,3-MIP_3 appears to be hydrolysed by enzyme IE and IC, whereas 2,3,4-MIP_3 by enzyme II. The phosphate monoester bonds of MIP_2 and MIP_1 appear to be selectively dephosphorylated by both IE and II enzyme. The role of enzyme IA is not clear but it seems to be related to the dephosphorylation of MIP_6 only as its concentration parallels the concentration of MIP_6 during germination period.

In conclusion it can be said that various phosphate monoester bonds in phytate molecule are preferentially hydrolysed by different forms of phytase, the lower molecular forms being more specific for the lower inositol phosphates.

6.6 SUMMARY

(1) The developmental pattern of multimolecular forms of phytase was investigated in germinating Cucurbita maxima seeds. The de novo synthesis of phytase in Cucurbita maxima seedlings starts after nearly 12 hours of soaking in water and progressively increases upto 7th day of growth and then starts declining in the later stages of germination.

(2) Phytase system consists of two main forms of phytase, phytase I having high molecular weight and phytase II having low molecular weight. Phytase I appears at 12 hours of germination and is sharply peaked at 180 hours after which it declines. Phytase II appears at 36 hour and goes on increasing progressively. It becomes dominant after 180 hours.

Relative concentrations of phytase I forms are related to the different stages of germination. Phytase IB is dominant in first 36 hours of germination but declines later. Simultaneously IA increases and becomes dominant at 48 hours of germination and later remains major enzyme upto 180 hours. The IE

gradually increases from the beginning (12 hours) and achieves considerably high activity at later stages of germination. Activities of IC and ID remain quite low during whole period.

(3) By the cycloheximide treatment of seedlings of Cucurbita maxima for 180 hours, the synthesis of all the phytase I isoenzymes was completely inhibited, whereas the inhibition of phytase II synthesis was reduced from 95 % to 77 % during the 84-180 hour period of germination suggesting that the phytase II may be a conversion product of phytase I.

(4) The role of each of the phytase species in the dephosphorylation course of phytate in germinating Cucurbita maxima seedlings has been proposed.

TABLE-16 : RELATIVE PROPORTIONS OF ACTIVITIES OF PHYTASE I AND I AT DIFFERENT STAGES OF GERMINATION OF CUCURBITA MAXIMA SEEDS

Period of Germination (hr)	Phytase I (Units)	Phytase II (Units)
0	-	-
12	75	-
36	171	6
48	188	44
84	213	68
180	288	90
228	145	131
300	127	153
372	78	177

Phytase I and II were obtained by Sephadex G-150 gel filtration of 30-80 % saturated $(NH_4)_2SO_4$ fraction of phytase.

TABLE-17 : RELATIVE ACTIVITIES OF PHYTASE ISOENZYMES DURING VARIOUS PERIODS OF GERMINATION.

Germination Period(hr)	Activity of Phytase I Isoenzymes					Total Phytase Activity (units/ 100 seeds)
	IA	IB	IC	ID	IE	
0	-	-	-	-	-	-
12	17(18%.)	38(41%.)	4(4%.)	3(3%.)	7(8%.)	92
36	39(20%.)	43(22%.)	6(3%.)	7(4%.)	26(13%.)	194
48	91(35%.)	13(5%.)	6(2%.)	32(12%.)	46(18%.)	260
84	98(31%.)	9(3%.)	30(10%.)	13(4%.)	47(15%.)	317
180	117(36%.)	8(3%.)	28(9%.)	11(4%.)	52(16%.)	327
228	61(19%.)	6(2%.)	7(2%.)	8(3%.)	49(15%.)	327
300	42(14%.)	5(2%.)	6(2%.)	6(2%.)	51(17%.)	300
372	10(4%.)	4(2%.)	3(1%.)	4(2%.)	49(19%.)	258

The isoenzymes of phytase I were separated by CM cellulose chromatography. The figures in parenthesis indicate the percent of the total phytase activity recovered. Total phytase activity expressed here is the total units of 30-80 %. saturated $(NH_4)_2SO_4$ precipitate of phytase obtained.

TABLE-18 : EFFECT OF CYCLOHEXIMIDE ON THE SYNTHESIS OF PHYTASE CUCURBITA MAXIMA COTYLEDONS AT DIFFERENT STAGES OF GERMINATION.

Period of Germination (hr)	Cycloheximide[1] Concentration (μ mole)	Total No. of Cotyledons.	Phytase Synthesis (Units) Control	Phytase Synthesis (Units) Treated	Percent Inhibition[2]
0- 12	50	100	92	0	100 %
12- 36	50	100	102	64	37 %
36- 84	50	100	123	-	100 %
84-180	50	100	10	-	100 %

[1]Cycloheximide treatment was given after 12 hr germination. One hundred germinating cotyledons were picked up at random and analysed for phytase activity.

[2]Inhibition of phytase activity is expressed as percent of control.

TABLE-19 : INHIBITION OF SYNTHESIS OF PHYTASE ISOENZYMES BY CYCLOHEXIMIDE TREATMENT

Enzyme	Total Phytase Enzyme Synthesized (Units)								
	Period of Germination								
	12-36 hr			36-84 hr			84-180 hr		
	Control	Treated	Inhibition[1] (%)	Control	Treated	Inhibition[1] (%)	Control	Treated	Inhibition[1] (%)
IA	39	13	66.7	59	-	100	19	-	100
IB	43	15	65.1	-	-	-	-	-	-
IC	6	4	33.3	24	8	66.7	-	-	-
ID	7	2	71.4	6	2	66.7	-	-	-
IE	26	12	46.2	21	-	100	5	-	100
II	6	2	66.7	62	3	95.2	22	5	77.3

[1]Percent Inhibition is expressed as percent of the units of the control.

Fig. 25 : PHYTASE ACTIVITY AS A FUNCTION OF GERMINATION PERIOD.

Phytase activity of $(NH_4)_2SO_4$ fraction (30-80 % saturation) extracted from cotyledons of Cucurbita maxima seeds germinated at various periods (0 - 84 hr) was determined. Total units of phytase activity per 100 seeds is plotted as a function of germination period in this figure.

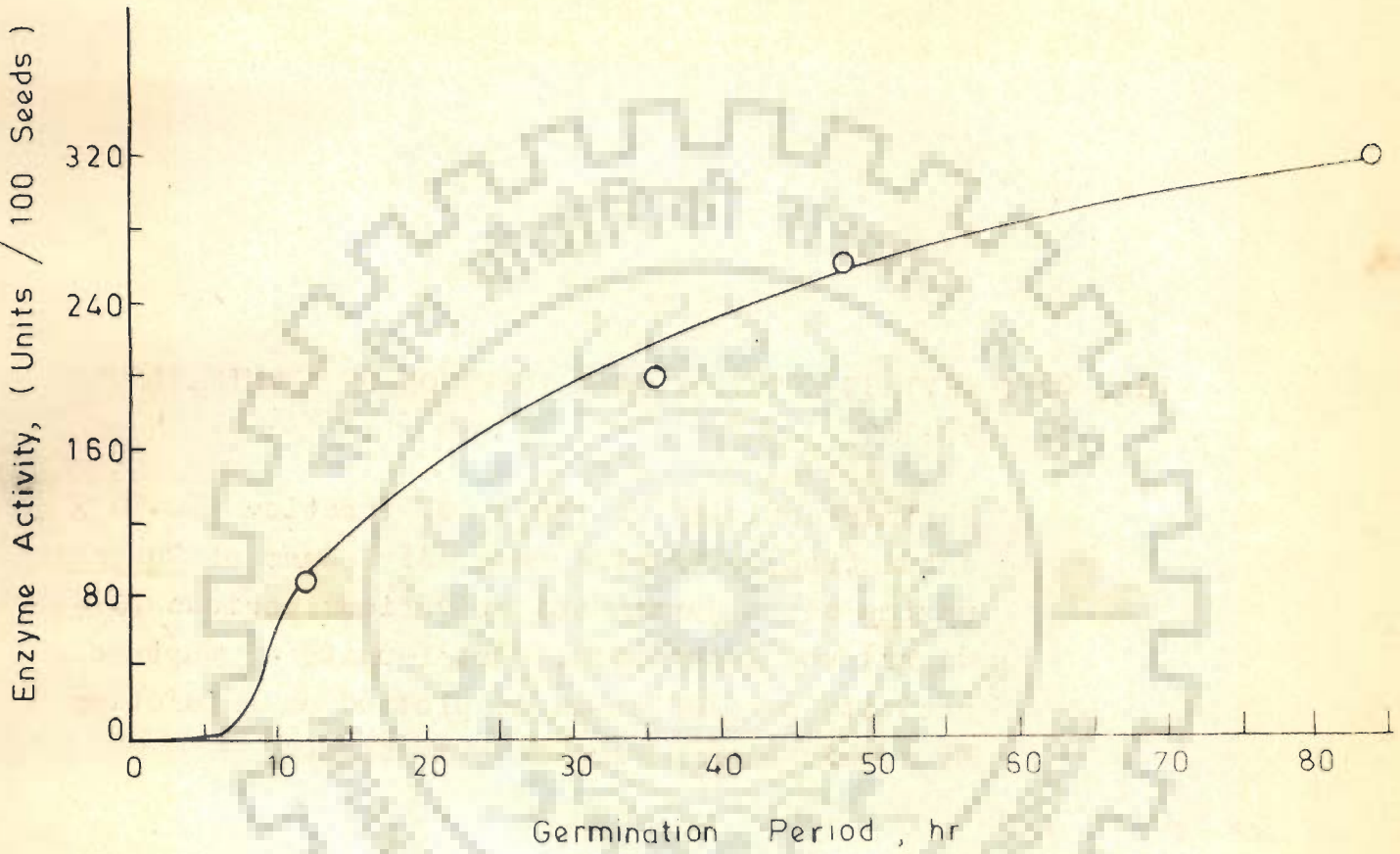


Fig. 25 - Phytase Activity as A Function of Germination Period

Fig. 26 : RELATIVE CHANGES IN PHYTASE I AND II ACTIVITIES DURING GERMINATION OF CUCURBITA MAXIMA .

Isolation of phytase I and II was carried out by Sephadex G-150 gel filtration of $(NH_4)_2SO_4$ fraction (30-80 % satn) of phytase. The enzymes were eluted from a Sephadex G-150 column (1x70 cm) by 0.05 M acetate buffer of pH 5 at a flow rate of 6 ml/hr. Each fraction, collected was of 2 ml. The elution profiles of phytase I and II during different periods (12 hr-372 hr) of germination of Cucurbita maxima are shown in this figure. ~~oo~~ represents activity and -•- protein concentration.

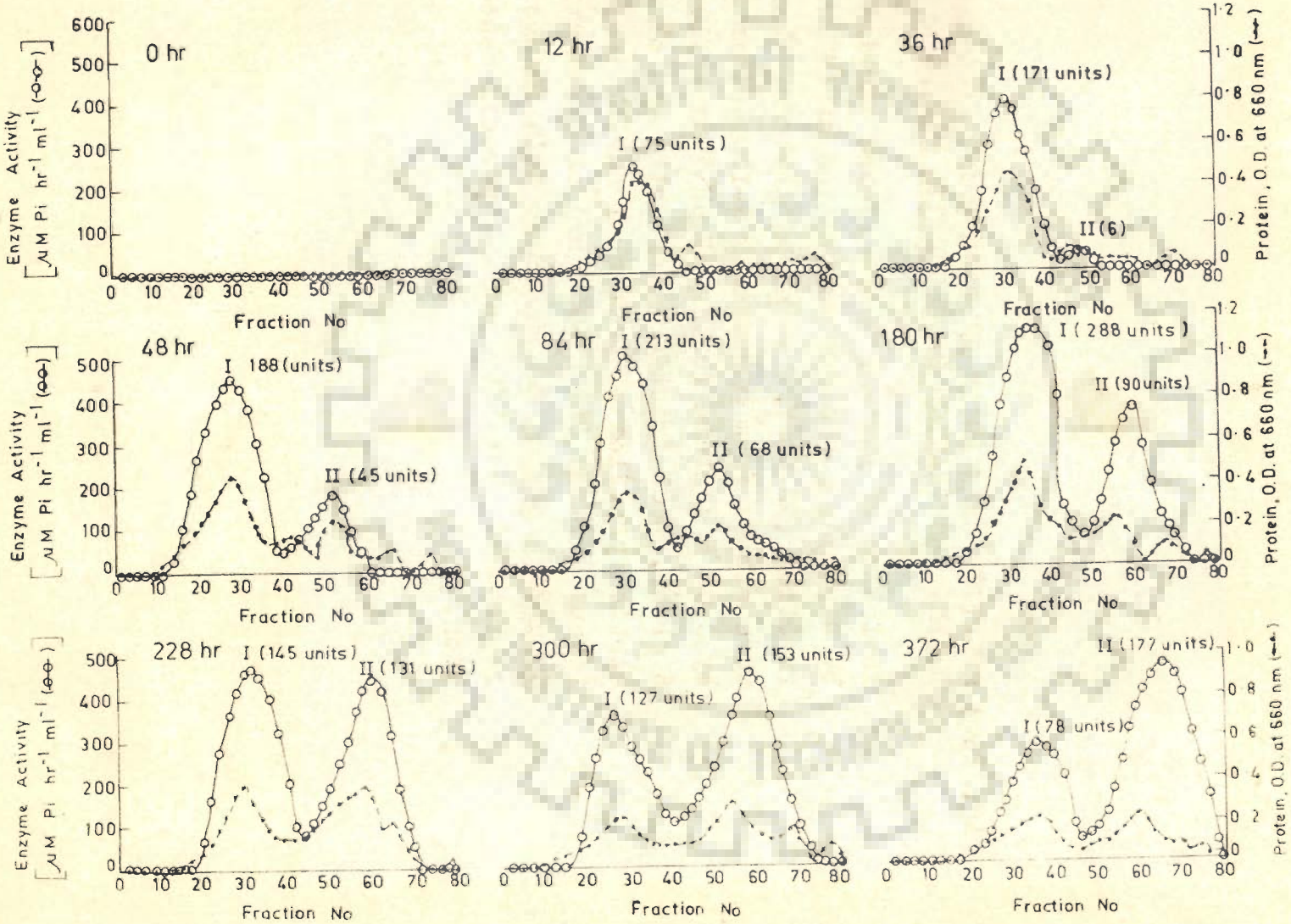


Fig.26 - Sephadex G-150 Gel Filtration Patterns of Phytases During Germination

Fig. 27 : RELATIVE CHANGES IN PHYTASE IA, IB, IC, ID AND IE ACTIVITIES DURING GERMINATION OF *CUCURBITA MAXIMA*

Phytase IA, IB, IC, ID and IE were isolated by CM cellulose column chromatography of peak I obtained from Sephadex G-150 gel filtration. The phytase species were eluted from a CM cellulose column (2 x 20 cm) by batchwise extraction of enzymes with NaCl gradients (0-0.7 M) in 0.05M acetate buffer of pH 5 at a flow rate of 0.4 ml/min. Fractions of 2 ml were collected. The relative changes in activities of phytase IA, IB, IC, ID and IE forms in cotyledons of *Cucurbita maxima* during different periods of germination (12 hr to 372 hr) are represented here. -●●- represents activity and ---- protein concentration.

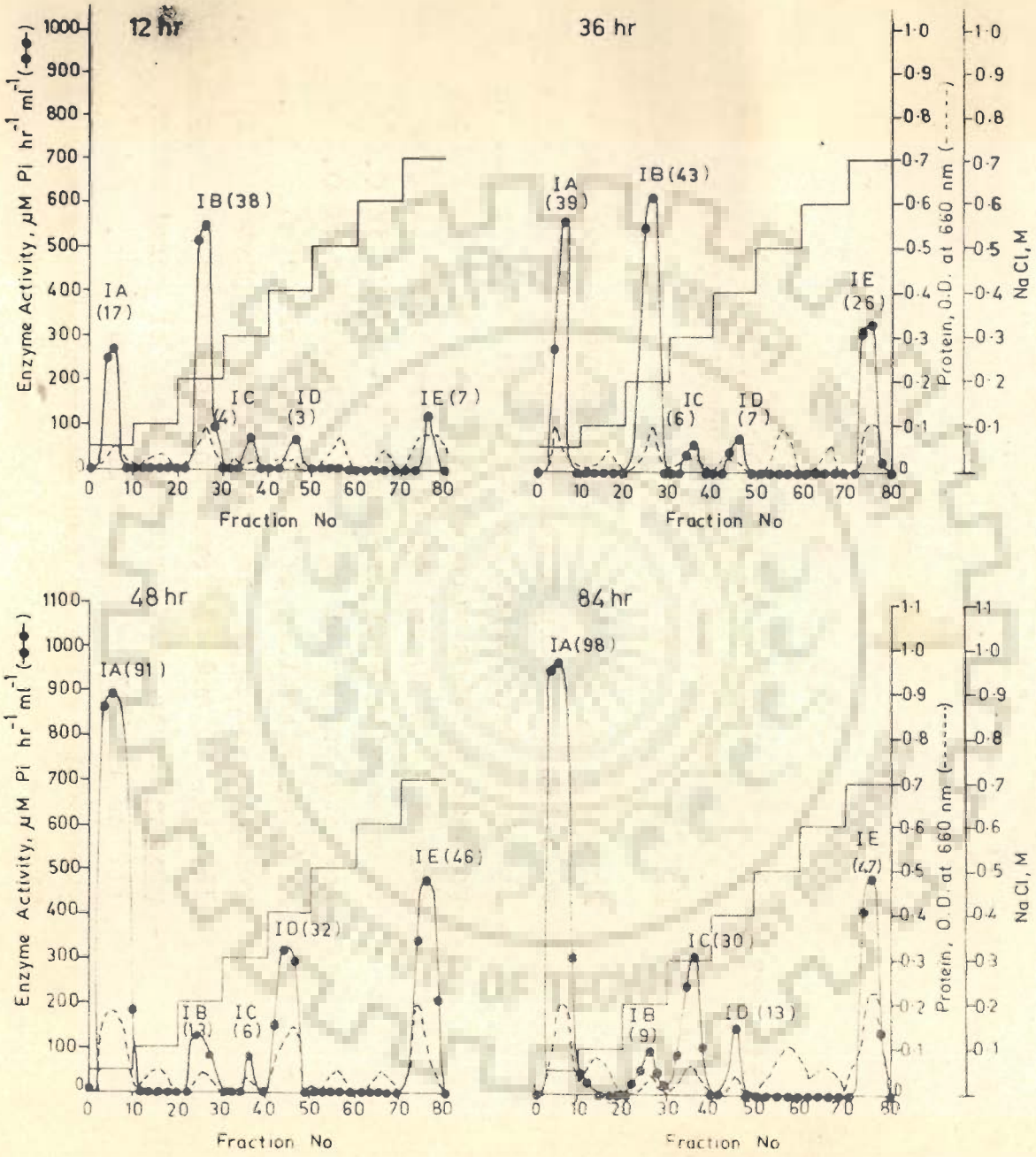


Fig27 CM cellulose Column Chromatographic Patterns of Phytases at Different Periods of Germination

(Numbers in parenthesis indicate units of enzyme)

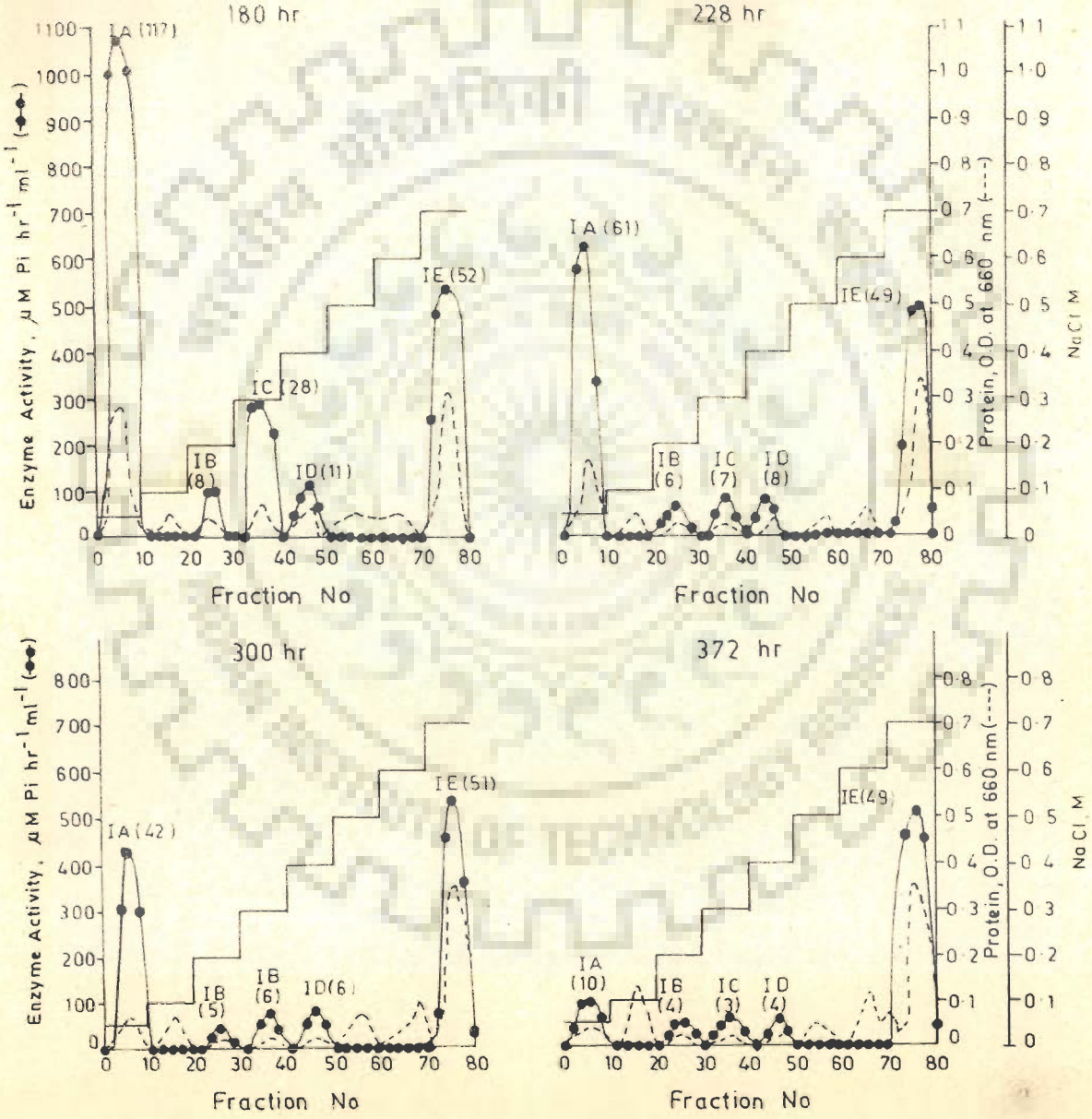
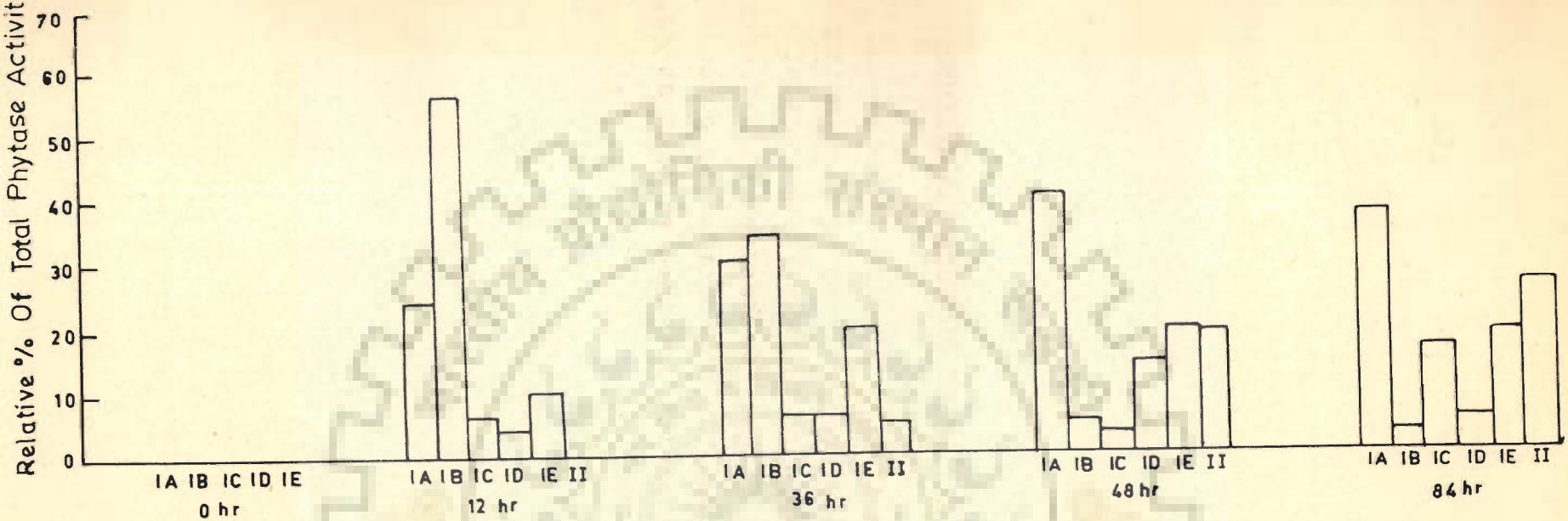

180 hr
228 hr
300 hr
372 hr
Enzyme Activity, µM Pi hr⁻¹ ml⁻¹ (●—●)
Protein, O.D. at 660 nm (----)
NaCl M
Fraction No
IA (117)
IB (8)
IC (28)
ID (11)
IE (52)
IA (61)
IB (6)
IC (7)
ID (8)
IE (49)
IA (42)
IB (5)
IB (6)
ID (6)
IE (51)
IA (10)
IB (4)
IC (3)
ID (4)
IE (49)

Fig. 28 : DEVELOPMENTAL PATTERN OF PHYTASE ISOENZYMES

Various phytase isoenzymes were isolated and purified from different stages of germination of Cucurbita maxima. In this figure activity of each phytase isoenzyme observed at different germination periods is represented as percent of total phytase activity obtained at that stage.


Relative % Of Total Phytase Activity
70
60
50
40
30
20
10
0
IA IB IC ID IE
0 hr
IA IB IC ID IE II
12 hr
IA IB IC ID IE II
36 hr
IA IB IC ID IE II
48 hr
IA IB IC ID IE II
84 hr
Germination Period Of Pumpkin Seeds

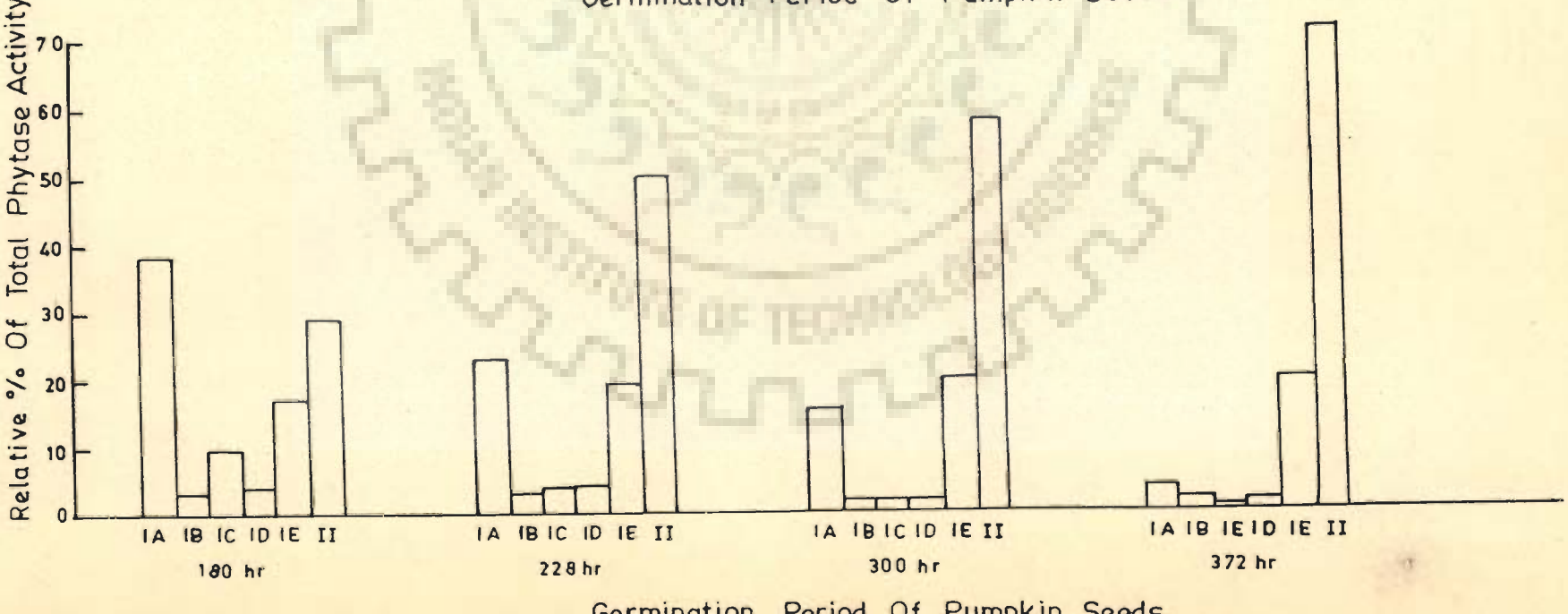

Relative % Of Total Phytase Activity
70
60
50
40
30
20
10
0
IA IB IC ID IE II
180 hr
IA IB IC ID IE II
228 hr
IA IB IC ID IE II
300 hr
IA IB IE ID IE II
372 hr
Germination Period Of Pumpkin Seeds

Fig. 29 : RELATIVE CHANGES IN ACTIVITIES OF PHYTASE I AND II DURING GERMINATION OF CUCURBITA MAXIMA IN PRESENCE OF CYCLOHEXIMIDE.

Isolation of phytase I and II was carried out by Sephadex G-150 gel filtration of $(NH_4)_2SO_4$ fraction (30-80 % satn) of phytase extracted from cycloheximide treated Cucurbita maxima seedlings. Enzymes were eluted from a Sephadex G-150 column (1 x 70 cm) by 0.05M acetate buffer of pH 5 at a flow rate of 6 ml/hr. Each fraction collected was of 2 ml.
This figure represents changes in phytase I and II activities during different periods of germination 36 hr, 84 hr and 180 hr in presence of cycloheximide. —— represents activity and -- protein concentration.

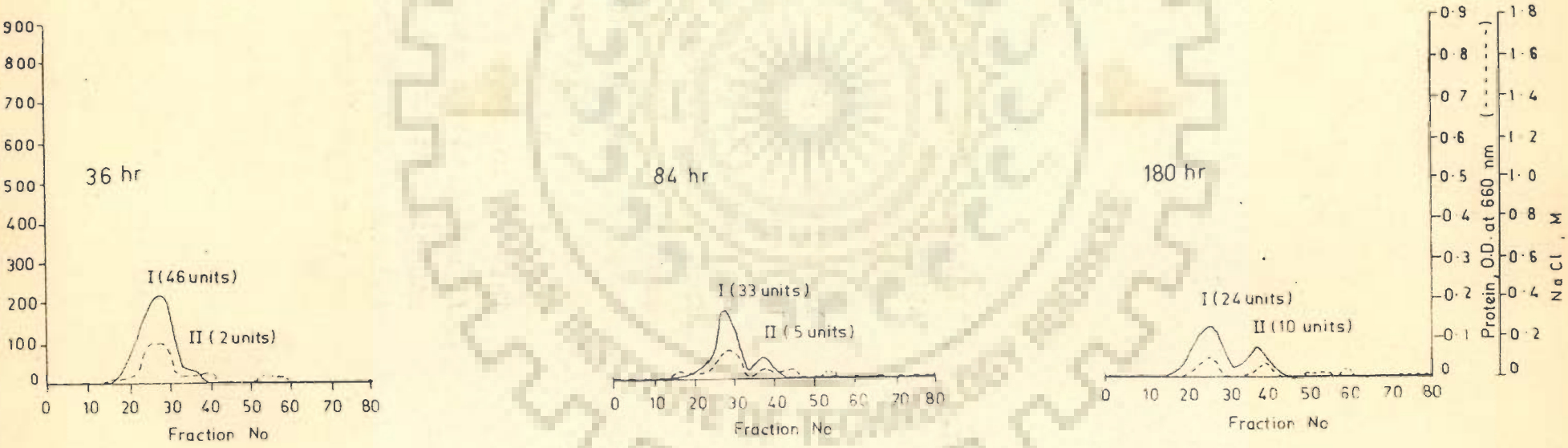


Fig. 29 - Sephadex G-150 Gel Filtration Patterns of Phytases in Cycloheximide Treated Cucurbita maxima Seedlings

Fig. 30 : RELATIVE CHANGES IN ACTIVITIES OF PHYTASE IA,IB, IC,ID,IE DURING GERMINATION OF CUCURBITA MAXIMA IN PRESENCE OF CYCLOHEXIMIDE.

Isolation of phytase IA, IB, IC, ID and II was done by CM cellulose column chromatography of peak I obtained from Sephadex G-150 gel filtration of phytase extracted from cycloheximide treated seeds. Phytase isoenzymes were eluted from CM cellulose column (2 x 20 cm) by batchwise extraction with NaCl (0-0.7 M) in 0.05M acetate buffer of pH 5 at a flow rate of 0.4 ml/min. Each fraction collected was of 2 ml. The relative changes in activities of phytase IA, IB, IC, ID and IE during 36, 84, 180 hrs of germination in cycloheximide treated seeds are represented in this figure. -oo- represents activity and --- protein concentration.

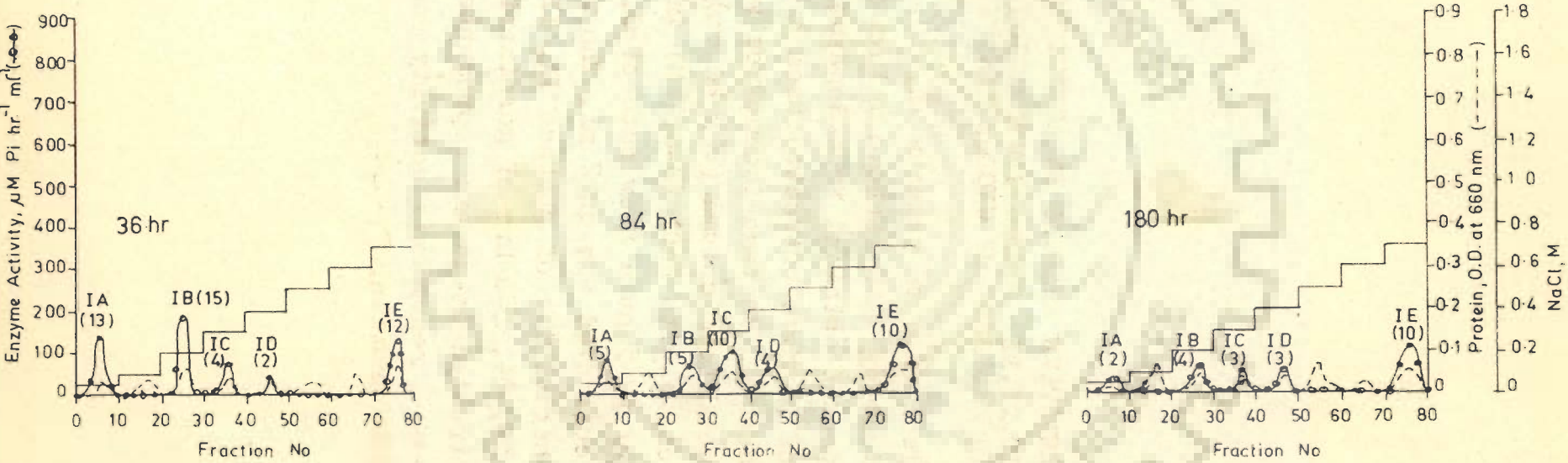


Fig 30 CM cellulose Chromatographic Patterns of Phytases in Cycloheximide Treated Cucurbita maxima Seedlings

(Numbers in parenthesis indicate units of enzyme)

Fig. 31 : EFFECT OF CYCLOHEXIMIDE ON ACTIVITIES OF PHYTASE ISOENZYMES AT VARIOUS PERIODS OF GERMINATION.

Total units of phytase isoenzymes in cycloheximide treated and untreated pumpkin seeds at various periods of germination are presented in this figure.

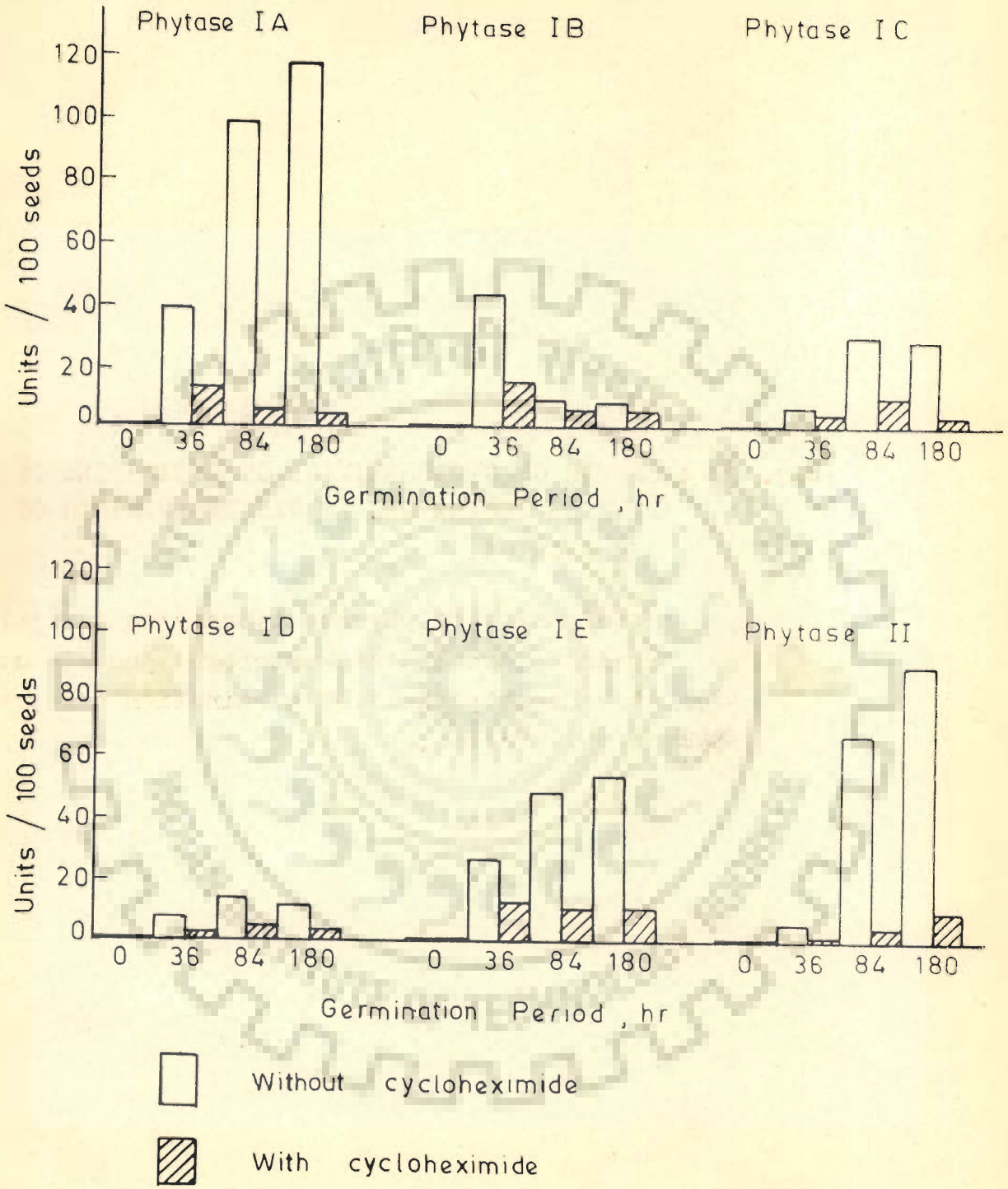


Fig.31 - Effect of Cycloheximide on Development of Phytases

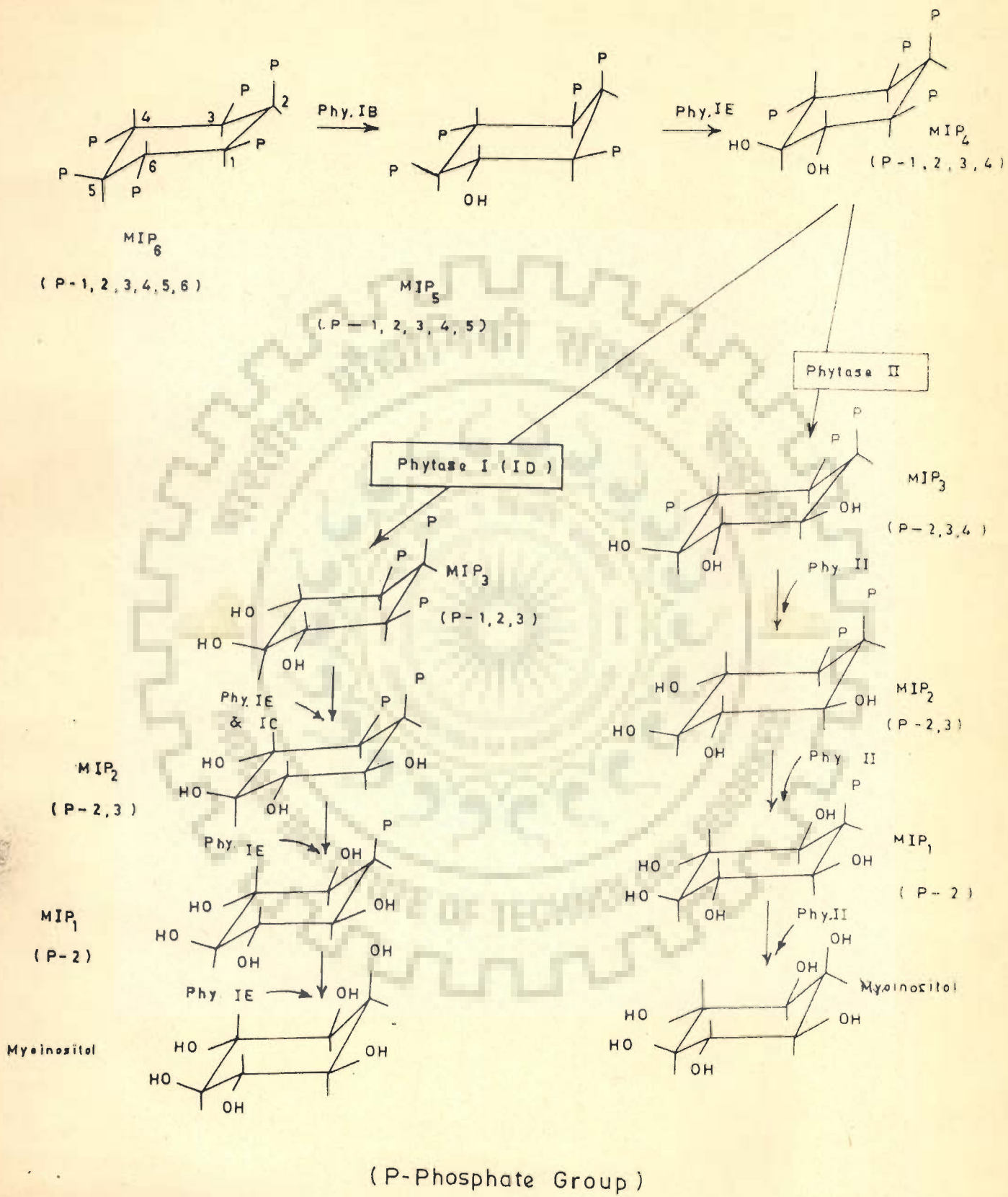
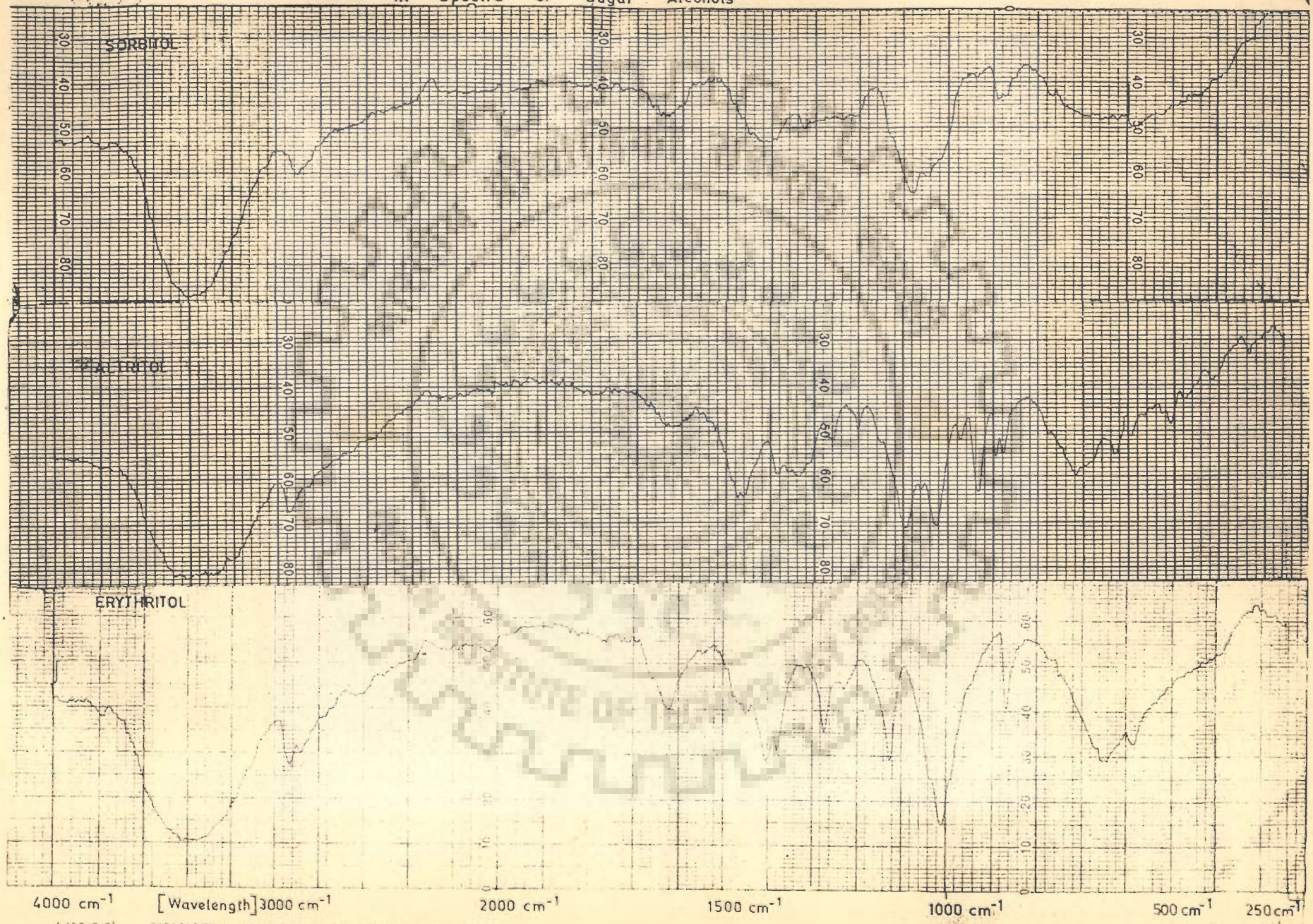


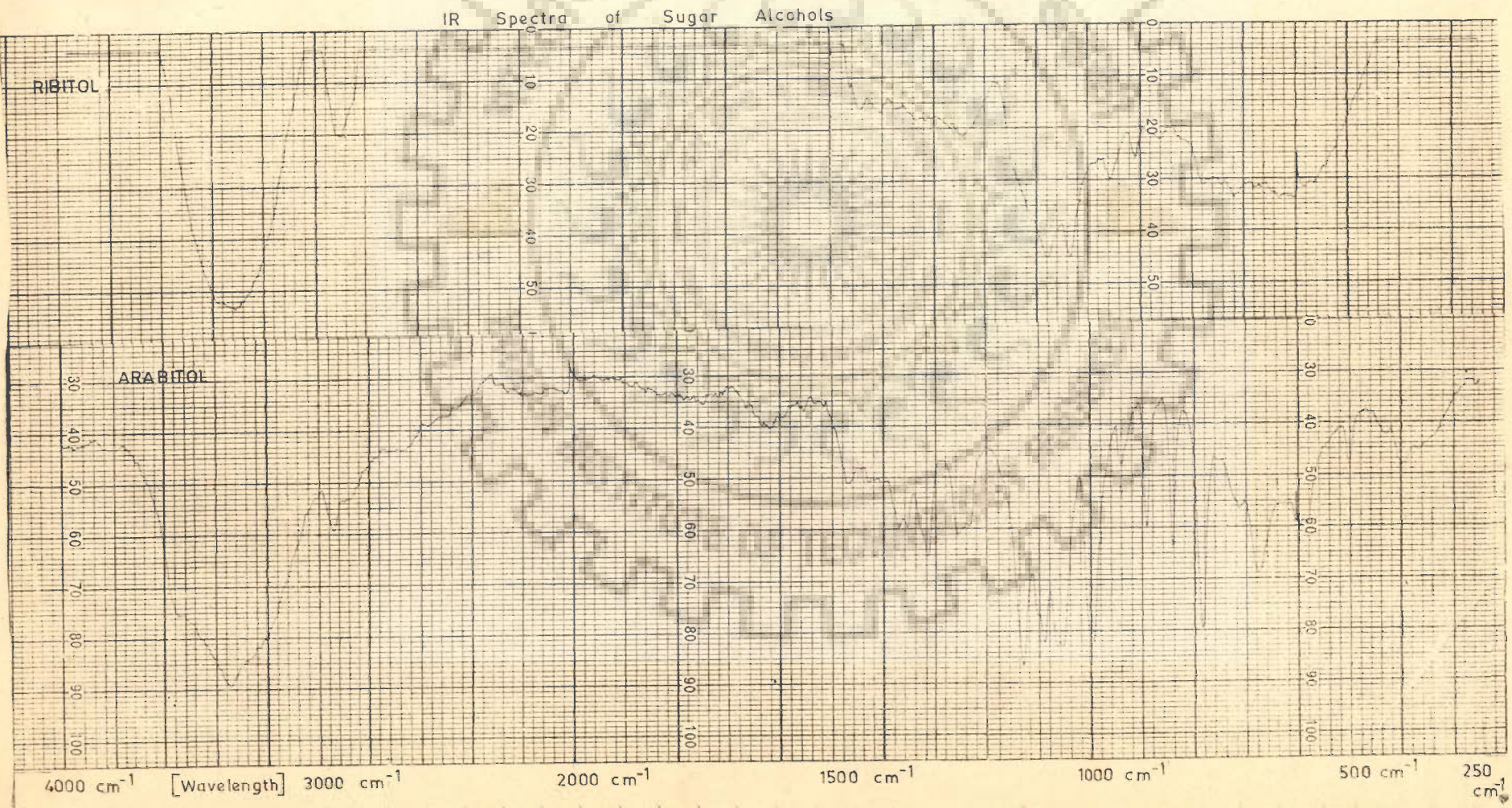
Fig.32 - Dephosphorylation Pathway of Phytic Acid

APPENDIX

TECHNICAL GRAPHS CORPORATION (I), NEW DELHI Chart

IR Spectra of Sugar Alcohols





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